

The stewardship of conscience

Researchers contributing to the 'stockpile stewardship programme' should face the truth about its potential role in maintaining US reliance on the use of nuclear weapons.

Recent efforts by US nuclear weapons laboratories to engage in deeper and broader collaboration with university researchers have encountered a number of practical obstacles, including the role of foreign faculty and staff in such partnerships (see *Nature* 391, 311; 1998). So far, however, they have not faced any serious resistance on university campuses to acceptance of the money.

Defenders of these partnerships have argued, with some success, that the research they will carry out will not support the development of nuclear weapons as such, but merely assist the weapons laboratories in their new mission of assuring the "safety and reliability" of existing weapons through the \$4.5-billion-a-year science-based stockpile stewardship programme. The problem is that this programme is not needed to ensure either the safety or the reliability of the existing stockpile. That is already quite safe and reliable, and could be kept so indefinitely by means of a small remanufacturing programme (see Ray E. Kidder, *Nature* 386, 645; 1997).

The real aims of science-based stockpile stewardship appear to be twofold. The first, which cannot be publicly acknowledged, is to maintain the three nuclear weapons laboratories at their Cold War level, so that Washington does not have to make politically painful choices. The second — also seldom discussed, but at least acknowledged in Department of Energy documents — is the long-term maintenance of a US nuclear weapons design capability.

These aims overlap, but are not identical. The laboratories at Sandia and Los Alamos are by far the main source of federal largesse in the state of New Mexico, whose senior senator, Pete Domenici, happens to chair the Senate's Budget Committee. The Lawrence Livermore laboratory is seen in northern California as a vital technological resource. It became clear early in President Bill Clinton's second term that the laboratory would not close or change its mission, whatever advice the administration received to the contrary.

The need for a future weapons capability compounds these blunt political considerations. The Clinton administration has a policy of

not developing new weapons at present, but the stockpile stewardship plan states clearly that the capability to do so must be maintained. Major facilities, such as the National Ignition Facility at Livermore, are intended to help realize that goal. So are the partnerships with the universities.

One of these, at the University of Utah, has kept critics at bay by arguing that it will model only issues pertinent to nuclear weapons safety. What, then, of the other partnerships that will model explosive shock, or turbulence — both problems more directly tied to bomb simulation? Scientists at all five university partnerships point out that they are doing unclassified work only in basic physics. But the objective of that work, from the funder's viewpoint, is to lay the groundwork for as complete a simulation of nuclear weapons as is possible in the absence of testing.

The university scientists also claim that, by supporting the stockpile stewardship programme, they are enabling the United States to comply with the Comprehensive Test Ban Treaty (CTBT), which most support. But the programme helps the CTBT only by injecting so much money into the weapons laboratories that their directors will desist from undermining the treaty in the Congress. And the argument that the programme reflects the will of the president and of the Congress, a political tapestry that patches together various special interests, is not a reasonable basis for a university to decide what it will and will not expect its staff and students to do.

Rather than telling the public the truth about the stockpile stewardship programme, however, the scientific community has put aside obvious questions and quietly accepted the money. In doing so, it has implicitly endorsed a military posture that remains heavily reliant on weapons of mass destruction. General Lee Butler, former chief of the Strategic Bomber Command, recently called for this posture to be changed. Scientists with a conscience should follow his example and suggest how nuclear weapons research can be curtailed rather than expanded. □

Time to sweeten a bitter pill

New Zealand needs to show renewed imagination if reforms of its science base are to succeed in the long term.

It would be a shame if lack of financial imagination on the part of the New Zealand government were to undermine the bold experiment in science funding that has been under way since the early 1990s. The exercise, whatever its successes, has been painful for many of those involved. Signs that government enthusiasm may be waning for the measures needed to take full advantage of the changes will do little to raise morale in the research community (see page 426).

The essence of the experiment involved severing the close links between the government and the research activities for which it had previously been directly responsible. This was achieved by placing the relationship on a 'purchaser/provider' basis, and included breaking up the former Department of Scientific and Industrial Research. The strategy has not been without its successes. Several of the autonomous 'Crown research institutes' that now act as the principal 'providers' of government-funded research have achieved healthy surpluses on

research contracts negotiated with both government departments and private industry. And the Marsden Fund, set up in 1994 to provide competitive, non-targeted grants, has done much to demonstrate that the country remains committed to some basic research.

But major problems remain. Some are a by-product of the new approach; an increasing reliance on short-term contracts has dented the attractiveness of science as a stable career. Others remain from before. So far, for example, the country's predominantly resource-based industries have shown little enthusiasm for increasing their investment in research. The challenge facing the New Zealand government is how to reap the benefits of the changes without destroying the future strength of its research base through excessive faith in either the effectiveness of market mechanisms or the political appeal of welfare spending. A new burst of imagination is called for; the country — and its researchers — deserve no less. □



Japan's research institutes face cuts in maintenance budgets

[TOKYO] Japan's structural reform of its national finances has left many of its research institutes facing substantial cuts in their maintenance budgets — even though the country is continuing to increase its spending on science and technology as part of a government plan to double spending on science between 1996 and 2001.

Although the overall budget for the 1998 fiscal year, beginning on 1 April, sees the smallest increase in recent years at 0.4 per cent, funding for science and technology has won a 4.9 per cent increase (see *Nature* 391, 111; 1998). The Ministry for Education, Science, Sports and Culture (Monbusho), whose overall budget fell by 0.4 per cent, appeared to maintain its support for research by increasing its science and technology spending by 5.1 per cent.

In practice, however, research institutes under Monbusho expect a difficult year ahead. The budget for the next fiscal year, which was approved by the Diet (Japan's parliament) this month, includes a 15 per cent cut in maintenance budgets. This is a result of the government's efforts to reform Japan's ailing financial system under the newly formed Fiscal Structural Reform Act.

Most of the large research institutes set up by Monbusho for joint university use, such as the National Astronomical Observatory (NAO), the Institute for Space and Astronautical Science (ISAS) and the National Institute of Genetics (NIG), will face such cuts, even though their overall research budgets remain the same as last year.

At NAO, which provides advanced observational facilities to researchers throughout Japan, the cuts would affect general maintenance of the facility, such as operation of the telescopes, and paying expenses and electricity bills.

Although the observatory has received substantial funding for its new projects, such as the construction of 'Subaru', the world's largest optical-infrared telescope, due to begin operation this summer in Hawaii, the budget cut would make it difficult to carry out new research at NAO.

"The situation is ironic — possessing all this sophisticated equipment will mean very little if we can't switch it on," says Keiichi Kodaira, NAO's director. "Without being able to maintain the facility, it would be impossible to carry out internationally competitive research."

Most institutes have started to plan cost-cutting measures for the next fiscal year, trying to minimize damage to their research projects. "We are still trying to plan various



Cold prospects: radio telescopes may lack enough funds to run.

measures for overcoming this difficulty," says NIG's director, Yoshiki Hotta. According to Hotta, the budget cut would mainly affect computer maintenance, species preservation and feeding of laboratory animals.

Officials at ISAS expect that the 15 per cent cut would affect basic research on the development of satellite launch vehicles. Atsuhiko Nishida, director of ISAS, is particularly worried that the cut would jeopardize future research, especially if all efforts are directed towards saving current research projects.

The problem lies with the government's general account, which supplies the national

institutes' maintenance budgets, and which has been cut across the board to meet the government's restructuring plan. Under the plan, government deficits must be reduced from the current level of 5.9 per cent to 3 per cent of gross domestic product.

Various government-related science ministries and agencies have managed to maintain growth in their overall budgets by expanding special accounts that operate separately from the general account. But, in the case of the national institutes, these budgets cannot be allocated to maintenance costs.

According to Nishida, the funding system is badly designed for supporting long-term research projects such as those being done at ISAS. "If the government is to fully implement the 'five-year plan' to support scientific research, they will have to create a system under which scientists are given an environment to carry out competitive research," says Kodaira.

Asako Saegusa

Green light for US spallation source

[WASHINGTON] US Vice-President Al Gore announced last week that the Clinton administration will include \$157 million in its budget request to Congress for the 1999 fiscal year (which begins on 1 October 1998) to begin building a new spallation source for neutrons at the Oak Ridge National Laboratory in Tennessee.

The Department of Energy (DOE), which operates Oak Ridge, has been planning to build the \$1.3 billion facility for some time, but the project has until now received funding only for the planning stages. When completed in 2005, it will be the most powerful source of pulsed neutrons in the world, and will help scientists to refine their characterizations of materials and biological structures.

Gore, a former senator and congressman from Tennessee, said the new facility "will help us to reclaim America's position as the world leader in a technology we invented". At present, world leadership in spallation neutron production is filled by Britain's ISIS, and France is home to the most productive reactor source of neutrons for research.

The spallation project was conceived as a form of consolation prize for Oak Ridge, which three years ago lost its Advanced Neutron Source, a proposed \$3 billion research reactor to have been fuelled by highly enriched uranium, because of tight budgets and concern about nuclear proliferation (see *Nature* 383, 207; 1996).

Unlike reactors, which produce neutrons through the fission process, spallation sources literally knock neutrons loose from heavy elements such as tungsten, using a stream of high-energy protons from an accelerator. They are largely free from the nuclear waste and other environmental concerns associated with reactors.

Materials researchers, structural biologists and other scientists from universities, national laboratories and industry are lining up to use the DOE's three other neutron sources: Oak Ridge's High Flux Isotope Reactor, the Intense Pulsed Neutron Source at Argonne National Laboratory, and Los Alamos National Laboratory's Neutron Science Center. The Energy Secretary, Federico Peña, told the Oak Ridge audience that about 800 researchers use these neutron sources each year, but many more would like to.

The DOE's other major neutron source, the High Flux Beam Reactor at Brookhaven National Laboratory, has sat idle since the discovery of a tritium leak from the reactor's spent fuel basin more than a year ago (see *Nature* 388, 503; 1997). Peña recently announced that he had delayed a decision on whether to restart the Brookhaven facility until after the congressional elections next November. Two Republicans from New York, Senator Alfonse D'Amato and Michael Forbes, have announced their opposition to reopening the reactor.

Dave Kramer

Brain-drain warning over UK lab facilities

[LONDON] Britain's government minister responsible for universities warned last week that unless research facilities are improved, an increasing number of Britain's brightest science graduates will seek research positions in the United States.

The warning came from Baroness Blackstone, who, before her appointment last year as a minister in the new Labour government's Department for Education and Employment, was master of the University of London's Birkbeck College.

It coincides with a series of meetings between the Treasury and other departments about the government's future strategy for science funding that are being carried out as part of a comprehensive spending review.

Giving evidence to the House of Commons Select Committee on Science and Technology, Blackstone said that, in her former position, she had frequently been "ashamed" of the conditions in which researchers in many universities work.

"We have to do something," she said. Otherwise, the long-term consequences for Britain would be fewer highly qualified people working at the cutting edge of research. "That would mean we would lose out in terms of exports and general prosperity."

It could also mean wasting "an enormous amount of potential" among young scientists keen to do high-level research. "People who want to do research degrees will go to the United States, not because there is more research money, but because the research facilities are vastly superior to what we can provide. There is already a vast gap, and it is



Blackstone: admits she was 'ashamed'.

very important that that gap does not get any bigger."

Blackstone also spoke strongly in favour of the dual-support system, in which financial responsibility for university research is shared between the universities themselves (out of grants received from the government) and the research council.

"If we were to transfer [all funds for research] to the research councils, we would deny universities the opportunity to support the many young scientists who may not find it particularly easy to get substantial grants out of research councils," she said. "Also, there are many indirect costs of research which it is very difficult to be precise about, and for which the dual-support system provides an important cushion."

The future of this system, and in particular of government funding for infrastructure costs incurred by research funded through the research councils, is high on the spending review's agenda. It was identified as an area in need of urgent attention by the National Commission on Higher Education, chaired by Lord (formerly Sir Ron) Dearing, in its report last summer.

Treasury officials on the review have recently heard an outline of the view of the Office of Science and Technology presented by Sir John Cadogan, director-general of the

research councils, as well as individual presentations from the heads of the six research councils. A summary of these views is due to be delivered to a ministerial-level Treasury panel this week.

It is widely thought that the outcome of the review, due to be published in the summer, will be to peg future increases in science spending close to the expected level of inflation. (Budget figures for 1998–99, released last week, show an increase of only 0.6 per cent, in line with the plans of the former Conservative government.)

But the key questions are likely to be who should control how this money is spent — that is, what should be the balance between the universities and the research councils — and what external sources of funding can be secured for the science base, particularly from the private sector.

At last week's select committee hearing, Blackstone endorsed the universities' widespread opposition to the extra overhead costs of research council projects being met out of a further transfer of funds from Britain's four higher-education funding councils to the research councils. Such a move would reduce the universities' control over how these funds are spent.

Her warnings were echoed by John Battle, the minister for science, energy and technology. In his evidence to the committee, Battle pointed out that such a move would reduce the flexibility of university vice-chancellors to target funding on promising new fields.

But Battle also warned of the consequences of asking the research councils to increase their overhead costs — Dearing has suggested these should rise from 45 to 60 per cent of the direct costs — without additional funding from the Treasury.

Although the Royal Society appears to have been relatively sanguine about accommodating such a shift by cutting funding for lower-priority research projects, Battle warned that the effects would be devastating. "We would have to impose a moratorium on all new grants and studentships," he said.

On a more positive note, Battle told the committee about the success of a two-year-old scheme to encourage joint private/public funding of university research equipment. Figures released two weeks ago showed that £35 million (US\$58 million) provided last year by the funding and research councils towards this Joint Research Equipment Initiative — about £13 million more than originally planned — was matched by external contributions of just under £45 million.

Applications for support from the scheme, which the government had initially planned as a one-off event but which is now to be organized annually, were heavily oversubscribed. □

German chemist sues over fraud allegation

[MUNICH] Guido Zadel, a former chemist at the University of Bonn, who has been accused of committing scientific fraud four years ago, is suing the *Land* (state) of Nordrhein-Westfalen for DM437,000 (US\$242,000) compensation.

Zadel's lawyer claims that, as a direct result of the accusations, he has been unable to find a suitable post, and that the sum represents the amount a chemist of his age and qualifications could expect to earn in industry over four years.

Zadel is also seeking DM200,000 in damages from a former co-worker who has said that he saw Zadel manipulating experiments. Zadel is challenging a decision by the university to withdraw his doctorate.

Zadel had claimed to have discovered that a static magnetic field could, even without the use of polarized light, induce 'chiral synthesis' — the stereo-selective synthesis of optically active organic substances — a discovery that would have

had enormous significance for the pharmaceutical industry. Doubts soon arose about his claims, however, as no other research group was able to reproduce them (see *Nature* 382, 104; 1996). Zadel admits that his methods were "far from being mature". But he rejects charges that some failed experiments had not been properly recorded, and denies scientific misconduct.

Part of his defence is that, although his own paper was withdrawn soon after publication, two subsequent papers by other researchers appear to provide some theoretical support for his ideas. Both conclude that chiral synthesis in a magnetic field is in principle possible.

But researchers at the University of Bonn's Institute of Organic Chemistry have no plans for further work on the topic. "The common opinion is that chiral synthesis in a static magnetic field is impossible in practice," says Karl-Werner Glombitza, dean of natural sciences. **Quirin Schiermeier**

FDA turns down moratorium demand on xenotransplants

[WASHINGTON] The US Food and Drug Administration (FDA) last week resisted calls that it should declare a moratorium on clinical trials of xenotransplantation on the grounds that more time is needed to find out about the potential risks.

The call for a moratorium has come from Fritz Bach, a xenotransplant scientist at Harvard Medical School, and eight coauthors, in an article to appear in the February issue of *Nature Medicine* (see *Nature Medicine* 4, 141–144; 1998 and *Nature* 391, 326; 1998).

At a two-day meeting convened at the National Institutes of Health (NIH) by the US Public Health Service and attended by leading administrators and scientists, Michael Friedman, acting FDA commissioner, said the call was a “highly valuable” part of the public debate. But he added: “We believe that it’s important to set up a framework to responsibly conduct research. And that’s what we’re endeavouring to do.”

The FDA wants to continue cautiously with trials with careful safeguards and strict supervision, and this position was reflected in the views of many experts at the meeting. “The field requires the beginning of well designed, well controlled clinical trials,” said Daniel Salomon, an immunologist at the Scripps Research Institute in La Jolla, California, and member of the board of the American Society of Transplant Physicians.

Salomon and others argued that the call for a moratorium was untimely, and demonstrated a lack of awareness of the public debate and regulatory process that has taken place over the past four years. Another critic was David Onions, professor of veterinary pathology at the University of Glasgow and author of a paper showing that pigs harbour endogenous retroviruses that can infect human cells *in vitro*.

The Bach article “was written as if none of us had ever thought of these issues,” said Onions, who sits on an FDA advisory subcommittee on xenotransplantation, and who opposes a moratorium. “It was as if [those calling for a moratorium] were unaware of what has happened.”

But some at the meeting supported Bach and his coauthors, who want a moratorium on clinical trials while a national committee of “broadly concerned citizens from many walks of life” grapples with the risk issues. Alix Fano, of the Campaign for Responsible Transplantation, based in New York, demanded: “Who will be held accountable if and when a zoonotic virus is spread to the human population?” Friedman acknowledged: “Those are difficult questions.”

Bach said he was not convinced by FDA

officials during the meeting that a ‘transparent’ process involving the public was important to the agency. The FDA and other regulators had done a “very good job” of drawing up regulations based on technical considerations. But he added: “I am not sure they are hearing the need for ethics to precede the technical discussion. And they have not focused on [the public involvement that] I and my coauthors feel should come first. That leaves me with great disquiet.”

Onions, Salomon and others contend that, in eight meetings over the past four years, US authorities have publicly debated the pros and cons of xenotransplantation, including the ethical issues. Guidelines for those engaging in xenotransplantation, issued by the Public Health Service in September 1996, and revised in a stricter form to be issued this year, reflect those years of careful debate, they say.

The revised guidelines, first described publicly at last week’s meeting but not final until published later this year, bring all xenotransplant trials under FDA’s oversight, rather than that of local institutional review boards. They include the setting up of a xenotransplantation registry and a central archive to house biological specimens for at least 50 years. FDA officials estimate the cost of the registry at \$250,000 to \$300,000 a year, and \$1 million a year for the archive.

The guidelines also suggest the creation of a National Advisory Committee. Mary Groesch, an NIH official who addressed the meeting on behalf of a Department of Health and Human Services committee on xenotransplantation, said that this should function much like the Recombinant DNA Advisory Committee, which gave public scrutiny to gene therapy protocols.

But Kathryn Zoon, director of the FDA’s Center for Biologics Evaluation and Research, said the agency is still developing its policy on how much data about proposed clinical trials would be made public before trials begin. She said the agency intends to make “a select subset” of information available “that will enable public knowledge of preclinical and clinical research”.

Jonathan Allan, a virologist at the Southwest Foundation for Biomedical Research in San Antonio, Texas, attacked the revised guidelines for failing to ban the use of non-human primates as donors or to establish stricter, separate criteria for their use.

Allan told the meeting that the health service has “ignored” a substantial risk, because the infectious disease risks to humans in the use of primate donors are far greater than those of pigs.

Meredith Wadman



NEAR view: a composite image of Antarctica, taken by the spacecraft’s multi-spectral imager.

Earth today, Eros in a year’s time, for asteroid spacecraft

[WASHINGTON] The NEAR (Near Earth Asteroid Rendezvous) spacecraft swung past Earth last week in a manoeuvre that puts it on target for an encounter with asteroid 433 Eros a year from now.

In addition to using Earth’s gravity to change the spacecraft’s trajectory, the swing-by gave project managers the chance to calibrate the craft’s instruments by pointing them at their planet of origin.

Among the pictures snapped by the craft’s Multi-Spectral Imager were a ‘family portrait’ of the Earth and Moon, and views of Antarctica and the surrounding ocean (above) taken after the closest Earth approach, which occurred 336 miles above southwest Iran. Sun glinting off NEAR’s solar panels made it visible to observers throughout the world as a steady light moving across the sky.

The spacecraft is expected to return its first, dot-like images of Eros next summer. On 20 December it will begin firing braking rockets in anticipation of the rendezvous, and will enter orbit around the 25-mile-long asteroid on 10 January 1999 at an altitude of 1,000 km above the surface.

The orbit will then drop to as low as 35 km during an intensive year-long study, which will include mapping the surface at high resolution and characterizing the asteroid’s bulk properties, mineral composition, geology and magnetic field.

The mission will end on 6 February 2000 with a slow ‘controlled crash’ on to the asteroid’s surface.

NEAR follows last year’s Mars Pathfinder as the second in the US space agency NASA’s Discovery series of low-cost planetary missions.

Built and operated by Johns Hopkins University’s Applied Physics Laboratory, it is the first planetary mission to be managed from outside the agency.

Tony Reichhardt

Genome unit falls foul of campus politics in India

[NEW DELHI] A dispute between faculty members and the vice-chancellor of the Jawaharlal Nehru University in New Delhi has blocked the establishment of a National Centre for Plant Genome Research on its campus. The move has embarrassed the Department of Biotechnology (DBT), which only a month ago had announced the launch of the centre with an initial investment of US\$7 million.

According to the department's secretary, Manju Sharma, the university was chosen because it already had a genetic engineering unit and a centre for plant molecular biology — both funded by the department. The plan was to merge these units with the national centre to create a strong centre for coordinating plant genetics research countrywide.

But opposition to the merger has come from the university's 400-member staff association, which complains that the creation of a non-teaching institute such as the planned centre would not benefit the university's students or researchers, and will only absorb funds that might otherwise come to the university from government.

At the centre of the controversy is vice-chancellor Asis Datta, a well known plant molecular biologist. The staff association argued that Datta reached the deal with DBT without the sanction of the academic council, and acted in "haste and secrecy" in transferring 15 acres for the centre in violation of a ban on the donation of university land to other institutions. It claims that the proposed centre is linked to the vice-chancellor's own field of interest, and that he and other select members of faculty "will be the only beneficiaries".

But Datta says the matter has been discussed and approved by the university executive council, of which he is the chairman. He says that merger of the two existing units — both facing closure in three months as their research projects come to an end — with the planned research centre was the best way to ensure continuity of jobs for the staff. He also argues that the new centre would work closely with university faculty members, and form part of a unique network of advanced plant genetic research in the country.

The dispute between the vice-chancellor and faculty members (who have been joined by the students' union) has now forced the ministry of education, which controls the university, to put plans for the new centre on hold. Admitting the setback, Sharma says her department is not insisting on an autonomous institute and will be open to "alternative suggestions" for establishing the centre.

K. S. Jayaraman

US 'split' over support for research project database

[WASHINGTON] The US government should provide \$3 million over three years to support a database that has been developed to track detailed information on all publicly supported research and development, according to a panel set up by the National Science Foundation (NSF) to assess the future of the database.

Rand Corporation, which set up the database for the government, has threatened to pull the plug on it on 1 March if does not get paid to maintain the system, which costs at least \$1 million a year more to run than it generates from user fees.

But as that deadline nears it remains unclear where the government will find the money to run the database, which is known as RaDiUS (for Research and Development in the United States). Some government agencies are said to be reluctant to support a management tool which they fear could be used to criticize their programmes and attack their budgets.

The NSF panel was chaired by Irwin Feller, head of the economics department at Pennsylvania State University. It concludes that "despite its weaknesses, it is extremely important that RaDiUS be continued," and warns: "If RaDiUS died because of a lack of funding, this would probably also kill any hope of any other such system for a number of years."

Alarmed at such a prospect, the staff of Representative James Sensenbrenner (Republican, Wisconsin), chair of the House Science Committee, and Senator Jeff Bingaman (Democrat, New Mexico) — two important supporters of science in Congress

— have been closely monitoring the fate of RaDiUS.

But a meeting last week between the NSF, the Office of Science and Technology Policy (OSTP) and the Office of Management and Budget (OMB) failed to make much progress in determining who should pay for the database. RaDiUS lost \$1.4 million last year after a plan to raise money from users of the system fell flat. According to one official who attended the meeting, NSF is not prepared to pay for the system out of its budget for the current year unless it receives a direct instruction from OSTP to do so.

RaDiUS was developed by Rand with the strong encouragement of Skip Johns, former associate director for technology at OSTP, who wanted the database as a tool to improve coordination of the government's \$70 billion research and development programmes. But Johns left OSTP in 1996, and officials there are now said to be unsure of its practical value.

According to Paul Herer, a planning adviser at NSF, RaDiUS lacks strong support from potential users in the government. The system suffers, he says, from receiving incomplete data on the activities of some agencies, and from being "unfamiliar" to possible users. "It's ahead of its time a little bit," Herer says.

But Holly Gwinn, chief of staff at OSTP, says that while "nobody considers RaDiUS to be a perfect system", its shortcomings would be "addressable" if the government decided that the database was worth having. "We've been working with OMB to see what the value added is," she says. "We haven't reached a consensus on this."

Colin Macilwain

Kennedy backed on use of tobacco money

[WASHINGTON] Senator Edward Kennedy (Democrat, Massachusetts), a long-time supporter of medical research funding, last week received the backing of advocates of biomedical research for a bill that would direct billions of dollars raised from tobacco companies to US biomedical research.

At a press conference in Washington DC last Friday (23 January), almost a hundred medical research societies endorsed the Kennedy bill, which would levy a tax of \$1.50 on every packet of cigarettes.

Just under 43 cents of that would be used to support biomedical and other research at the National Institutes of Health, the National Science Foundation and other federal agencies.

Kennedy estimates that research would receive an extra \$8 to \$10 billion each year



Kennedy: seeking boost for research.

under his bill, and says that such sums would "bring major dividends in the battle against disease". Donald Coffey, professor of urology at the Johns Hopkins University School of Medicine and president of the American Association for Cancer Research, said that the

bill funded research "at a level consistent with devastation of the cancer caused by tobacco use".

The bill is one of four to be introduced in the Senate as starting points for legislation codifying a \$368.5 billion settlement with the tobacco industry.

Meredith Wadman

NASA 'needs better analytical equipment'

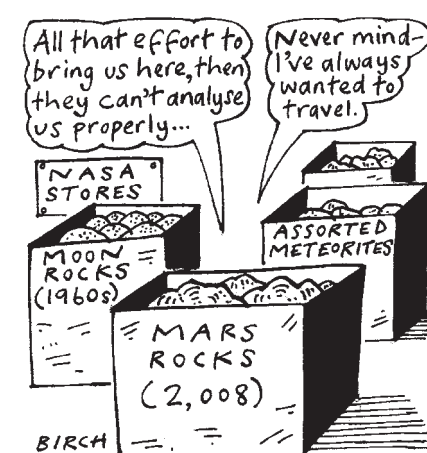
[WASHINGTON] The US space agency NASA must invest in new ground-based laboratory equipment if it wants to get the most out of samples returned from Mars and other extraterrestrial bodies in the next decade, says an advisory group.

By the time Mars samples are returned to Earth in 2008, most NASA-funded equipment will be at least 20 to 25 years old and obsolete, unless there is a major effort to upgrade, says the group.

Michael Drake, director of the University of Arizona's Lunar and Planetary Laboratory, says the present mass spectrometers, ion microprobes, electron microscopes and other equipment used to study extraterrestrial samples may not be up to the job.

Drake headed a task force asked by NASA's science office to look into the requirements for Laboratory Instrumentation for Analysis of Returned Samples (LIFARS). NASA invested heavily in laboratory facilities at the time of the Apollo programme in the 1960s, when hundreds of pounds of rocks were returned from the Moon. But since then there has been a "catastrophic decline in supporting infrastructure", says Drake.

Scientists studying meteorites and other extraterrestrial samples have worked around the problem in recent years by borrowing time on expensive equipment funded by the National Science Foundation



(NSF) and other agencies. But access is limited to scientists with NSF grants, he says.

The answer, according to Drake and other planetary scientists, would be a modest investment by NASA of around \$13 million a year in facilities for agency-funded researchers. The money would also help develop technologies such as resonance ionization mass spectroscopy, which promises greater precision in identifying isotopes. The NASA spending, if approved, would be coordinated with that of the NSF, the Department of Energy, universities and industry, all of which use similar equipment for different purposes.

No extraterrestrial samples have been

brought back to Earth for more than 20 years, but several such missions are planned. NASA's Genesis spacecraft will collect samples of charged solar wind particles and return them to Earth in 2003. In 2006, Stardust will return interstellar dust particles and dust from a comet's coma, and the Japanese-US Muses-C spacecraft will return material from an asteroid. Most ambitious will be the Mars sample return in 2008.

The Mars mission poses special problems because of the remote possibility that the returned rocks could be biologically active, or that they could be contaminated by terrestrial microbes once on Earth. To avoid contamination, NASA may use a single, ultraclean national laboratory for Mars rock analysis.

A task force headed by Michael Carr, a planetary scientist at the US Geological Survey in Flagstaff, Arizona, has begun looking into overall requirements for receiving, distributing and studying samples from Mars. A separate panel of the National Research Council's Space Studies Board is considering contamination issues related to small bodies such as comets and asteroids.

Drake says a full LIFARS programme is unlikely to appear in next year's NASA budget — to be unveiled in the Clinton administration's budget request next month — but might be included in 2000. **Tony Reichardt**

Germany's institute for scientific film could face final curtain

[MUNICH] Germany's only institute for scientific film, the Institut für den Wissenschaftlichen Film (IWF) in Göttingen, is threatened with closure following the termination of its government contract, depriving it of half its budget.

The move by the government last month symbolizes the tough approach being taken by the research minister, Jürgen Rüttgers, to establishments judged by the Wissenschaftsrat, Germany's science council, to be performing poorly (see *Nature* 387, 643; 1997).

As a 'blue-list' institute, the IWF is funded jointly by federal and regional governments. In the past, political pressure from the host region has meant that poorly performing institutes have proved difficult to close. But new institutes can be added to the blue list only if others are removed, and several institutes are keen to become members. At the top of the list are the Berlin-based Electron Storage Ring, a synchrotron radiation source, and the Institute for New Materials in Saarbrücken. Both should be added to the blue list as soon as possible, according to recent

recommendations by the Wissenschaftsrat.

The IWF acts as an audiovisual service institution for Germany, and its films and videos cover the full range of science from human ethnology to cell biology. Its archive of 6,600 films includes pioneering cinematographic works from the nineteenth century, such as the earliest studies of human movement disorders and film of the flight of cannon-balls. The IWF's most valuable documents are irreplaceable film of now-extinct species and tribes.

But in 1996 the Wissenschaftsrat concluded that the IWF had neglected developments in digital recording, and said that its lack of competence in, for example, electronic transmission of visual material suggested that it was unprepared for the global market. Despite several attempts, encouraged by the research ministry, to develop a new strategy, the institute was unable to convince the ministry to reverse its decision to withdraw its funding.

Ethnology and anthropology will suffer most if the institute closes, as "we are more dependent on films than natural sciences are", says Paul Henley, director of the

Granada Centre for Visual Anthropology at the University of Manchester. Similar institutions in Japan, the Netherlands, Hungary and Austria have been closed over the past ten years, he says. "With only a very few scattered institutes remaining it would be a great pity for anthropology worldwide if the IWF closed."

The IWF has one final chance to return to the blue list. The research ministry has promised to consider a new strategy, based on halving the institute's staff. The staff distrust the offer, however, believing there is no longer the political will to keep the institute open.

It remains unclear what will happen to the archived films if the IWF closes. Lower Saxony, where the institute is located, has its own state archive which could possibly serve as a depository. But scientists do not consider this a good option as it would mean leaving the recordings in the hands of non-experts, without production, distribution and marketing skills.

Lower Saxony has not indicated whether it will take over the full running costs of the institute or let it close. **Quirin Schiermeier**

New Zealand puts its science to profit

Peter Pockley

Six years ago the New Zealand government decided to make a number of radical changes in its approach to science funding. Supporters and critics remain divided on the outcome so far of its new strategy.

[WELLINGTON] Six years after the government of New Zealand introduced sweeping changes in its handling of science — the most dramatic being the dissolution of the 66-year-old Department of Scientific and Industrial Research (DSIR) — there are mixed views about their overall effect.

Even though New Zealand has slipped down the scale of science funding in international terms, supporters of the changes point out that research is now more tightly targeted on areas likely to benefit the country.

But many science and university leaders are concerned that, after several years of gradually increasing research budgets, the government may 'cap' its support this year, undermining recent gains and triggering disillusion about its commitment to research.

The changes followed a deep cut in funding for research and development (R&D) among many areas of public spending dur-

ing the country's economic crisis in the mid-1980s. This crisis persuaded the government to restructure the system of public services, with almost all public utilities and services being either privatized or corporatized as 'State Owned Enterprises'.

Designed by a Labour government and implemented by its National Party successors, the new system separates public organizations into three types of bodies: 'policy organizations', such as ministries and other government bodies; 'purchasing organizations', which provide funds; and 'providers', which in science are research organizations.

The application of these principles to the public science sector led to ten specialized Crown Research Institutes (CRIs) being set up as private companies out of the dismembered parts of DSIR and other government research units supporting the dominant agricultural industries (see below).



Mind the gap: Researchers' study characteristics of the Edgcombe earthquake in March 1997.

The changes involved large-scale job transfers and job losses among 4,000 staff, and cost NZ\$42 million (US\$24 million). There has also been a marked shift in employment in CRIs from research staff to non-scientists. In the five years to 1995–96, the number of researchers fell by almost 300 to just over 1,300 full-time equivalent staff, while support staff tripled to more than 900.

With research now seen as a 'commodity' to be 'bought' by purchasing organizations from research institutes — and supposedly subject to competitive pricing — New Zealand's science is tightly controlled.

Officials extol their approach as a model for other small countries. Even critics who describe the changes as "an uncontrolled experiment" admit that there has been a gradual upward trend in budget allocations.

But there are signs this rise will level out in 1998. And some scientists fear that the coalition government will cut spending on R&D by as much as NZ\$40 million to fund promised reductions in taxation and provide extra resources for the more politically popular fields of health and education.

Total government funding for R&D still amounts to NZ\$591 million in this financial year, including about NZ\$95 million for university research. But the Minister for Research, Science and Technology, Maurice Williamson, admits that future prospects for science and technology are not good.

Although "there will be no reduction in government research spending," he says that "baselines won't grow", and points out that the government would need to spend an extra NZ\$25 million on research in this year's budget, due in May, to keep up with gross domestic product (GDP), currently growing at about four per cent a year. This is seen as a warning that the extra funds will not materialize.

In international terms, New Zealand's support for R&D keeps it near the bottom of

'Purchasing' policy still stirs heated debate

In New Zealand's new system for supporting science, Crown Research Institutes (CRIs) operate as commercial companies, with their own boards and two government ministers as 'shareholders'.

They must attain 'profit targets' — surpluses of government grants and 'private' earnings over expenses — set by the government, which are then retained for investment.

The 'purchasing' of science is carried out by the Foundation for Research, Science and Technology (FRST). It distributes the so-called Public Good Science Fund (PGSF), rather like competitive grants bodies elsewhere except that it is told by the government how to divide up its funds, currently NZ\$282 million (US\$161 million).

Grants are allocated by panels according to priorities

set for 17 categories linked to commercial, industrial, agricultural, social or environmental sectors. These have now been joined under the jurisdiction of Williamson by the NZ\$24 million health research grants.

As the largest recipients of PGSF support, with NZ\$230 million this year, the nine CRIs depend on FRST for funding, which must be competed for every two years. Selection depends on research being cost-effective and relevant to national needs.

Critics say this resulted in a short-term, applied focus of research funding. But Stephen Thompson, FRST's new chief executive, describes the fund as "an investor in science".

Sean Devine, director of the Association of CRIs, challenges descriptions of the system as a market, saying it is "contrived, far from ideal

and extremely one-sided".

He believes pricing may shift successful grant applications from the highest quality towards "a greater volume and mediocrity". But Andy West, who helped set up the system, describes the reforms as "a solid success".

West was recently appointed chief executive of the Institute of Geological and Nuclear Sciences Limited (IGNS), the smallest institute with a 1996–97 budget of NZ\$24 million. It won research contracts worth \$17.9 million from government last year, the rest of the income coming from the private sector. It retained its operating surplus of NZ\$564,000, rather than repaying it to the Treasury.

Williamson's predecessor, Simon Upton, says the CRIs will not be privatized. But West queries "the whole notion of governments owning research institutes".

P.P.

members of the Organization for Economic Cooperation and Development (OECD). Public spending in 1992–93, when the restructuring took place, accounted for 0.59 per cent of GDP; three years later, it had slipped to 0.52 per cent, compared with an OECD average of 0.69 per cent.

Simon Upton, Williamson's predecessor, committed the government to a target of increasing this to 0.8 per cent by 2010. But Williamson says there has been "a loss of 'security of tenure' in our coalition government". And even Upton now admits that the prospects of a full government commitment to what he sees as an investment in the future "are in the balance".

Also under threat is another promise made by Upton that NZ\$30 million would be provided next year to the Marsden Fund, a scheme launched in 1994 that provides competitive grants for basic, non-targeted research in universities and CRIs.

Ross Moore, chief executive of the Royal Society of New Zealand, which administers the fund on behalf of the government, believes this goal is "in jeopardy". Indeed, with the value of the fund now standing at NZ\$22 million a year, and only one applicant in 13 being successful because of the fierce competition, any increase this year is unlikely to be more than NZ\$1 million. Sir John Scott, the society's president, describes the reduced funding as "a serious blow".

In a speech two months ago, Sir Ian Axford, who chairs the Marsden Fund, confirmed his support for the 1992 reforms, arguing they were necessary because the government had lost confidence in the former leaders of public research bodies.

But, while describing the system as "fairly reasonable", he argued that it was "ponderous and too concerned with process rather than purpose". Axford also criticized the policy of allowing science to be driven by market forces.

He also said the government must intervene to identify research priorities: "Unfortunately the level of our technology is rather low, and we desperately need to do something about it".

Scientists, said Axford, were now "too easily treated as seasonal workers who can be dropped and picked up on demand." Jobs were subject to individually tailored contracts, often limited by short-term grants.

One result is the difficulty for young scientists in establishing research careers, many having to settle for technician-grade posts that provide no chance of winning grants from the so-called Public Good Science Fund in competition with established scientists.

Megan Ogle-Mannering, for example, who holds a PhD in plant ecology from Otago University, recently left New Zealand after trying unsuccessfully for two years to find a post in which she might apply her training. She is now studying science com-

Government under fire from academics

To the disappointment of researchers, Maurice Williamson, New Zealand's Minister for Research, Science and Technology since 1996, has dropped the practice of his predecessor, Simon Upton, of consulting widely and directly with scientists, depending solely on policy advice from the 34 members of his ministry.

Where specific studies are needed, they are usually commissioned from the Royal Society of New Zealand under contracts worth NZ\$1.4 million this year. Established in an act of parliament, which was recently revised to extend its coverage from traditional to social and applied sciences, the society is officially independent of the government.

But maintaining its autonomous role is a delicate matter. In a celebrated spat starting in 1995, Philippa Black, a geologist and then president of the society, was declared *persona non grata* by Upton for criticizing the science system he championed (see *Nature* **379**, 112 & **380**, 282; 1996).

Black says: "If anything my views have strengthened since then with the current strain on funding, including the impossible infrastructure position of PGSF, and



Black: critical of new system.

Marsden grants not allowing purchase of equipment costing more than \$5,000, and the high costs of managing research."

Sir John Scott, recently elected as the society's new president, describes the government's position on science as technically amounting to cuts, which he finds "very depressing". He says he runs the same risk as Black in describing the restructuring of the economy and science as "experimental and too dependent on accountants".

George Petersen, president of the academy council of the society and a biochemist at the University of Otago, says that one of the problems in New Zealand science is how to boost the morale of scientists, which he describes as being "at an all-time low", especially in universities.

He sees the 'technology foresight' process planned by

the government (see below) as not being properly linked to funding, educational and scientific workforce requirements.

In a joint statement, Scott and Petersen say they urgently need to lobby the government to influence long-term policy "without being continually knocked back with the response that scientists are just looking after their own interests".

The New Zealand Association of Scientists (NZAS) is less cautious. Its president, Brion Jarvis, a retired microbiologist, says he was elected because of his advocacy of science and his ability to comment freely, now that he is no longer bound to any organization.

Evenly split between universities and CRIs, the NZAS speaks for CRI staff who are prevented by their contracts from speaking publicly on policy issues.

Jarvis says there is a "groundswell of discontent" among CRI scientists and uncertainty over jobs. Indeed a US Fulbright scholar, Jack Sommer, found in 1995–96 that only a quarter of CRI researchers felt they could speak freely on public policy issues. Most said their job satisfaction has decreased in the past two years. **P.P.**

munication in Australia.

A further problem is the continuing lack of investment in R&D from the previously highly protected private industry sector. Those responsible for introducing the new system had promised initially that it would stimulate such investment. But it remains stubbornly low, standing at only 0.26 per cent of GDP in 1995–96; indeed, a survey last year found that expenditure declined from NZ\$248 million in 1993–94 to NZ\$240 million in the following year.

Williamson and the new prime minister, Jenny Shipley, have both acknowledged the urgent need for New Zealand's industry to become more competitive internationally, but are aware of the difficulties. Both place their hopes in a two-year consultative study of national goals and needs using the techniques of 'technology foresight' (see *Nature* **390**, 651; 1997).

By using focus groups and media publicity, the government hopes to stimulate public enthusiasm for science, in the belief this will lead to an increased emphasis on research in both public and private investment and will help shift priorities within science to the needs of high-technology industries.

One important barrier to such a change in attitude appears to be the powerful Treasury, which remains opposed to government intervention. But government officials say they are optimistic about the new strategy.

Jill White, the Labour opposition spokeswoman for science, also says she welcomes the 'foresight' study but "only if the commitment to extended funding to reach the 2010 target is delivered". Williamson's statements, she says, appear to show that the government is "reneging with a weakening commitment to science". **Peter Pockley**

Germany to cut red tape surrounding genetic research

[MUNICH] Germany's research minister, Jürgen Rüttgers, has submitted new proposals to the German cabinet simplifying the conditions under which genetic research can be carried out. Following recommendations made by the Research, Technology and Innovation Council last year (see *Nature* **387**, 7; 1997), Rüttgers said that the lowest of the four safety levels as defined in Germany's gene technology law should be abolished.

The proposed change would enable researchers to carry out routine laboratory work, as well as field trials with genetically modified organisms, without going through lengthy bureaucracy. "According to the council, there is no basic risk specifically involved in gene technology," Rüttgers said in Bonn last week.

Europe's ban on growth hormones wins support

[LONDON] The World Trade Organization (WTO) has backed a European Union (EU) ban on the use of growth hormones in meat production, reversing a decision made by a WTO panel last August. However, the WTO

rejected the EU's claims that its ban was based on a scientific risk assessment.

A WTO appeals body ruled that the EU has a right to establish on a scientific basis a level of consumer protection that it considers appropriate and that is higher than international health standards.

Historian takes top job at science council

[MUNICH] Winfried Schulze, a historian at the University of Munich, was elected last week as the head of Germany's influential science council, the Wissenschaftsrat. He succeeds Dagmar Schipanski, an electronics engineer, who was the first woman president of any German research organization.

The Wissenschaftsrat, founded in 1957, serves as an independent advisory council for research and university policies. Its 54 members include 24 outstanding scientists and eight public figures, all appointed by the German president. The remaining council members are representatives of the *Länder* (states) and the federal government.

Chirac tries to broker deal on nuclear test ban treaty

[PARIS] There was a hint last weekend of an initiative to ease the current impasse over the entry into force of the Comprehensive Test

Ban Treaty (CTBT). Jacques Chirac, the French president, suggested that international cooperation on nuclear safety could be offered to India in return for a softening of India's refusal to join either the CTBT or the earlier nuclear non-proliferation treaty (see *Nature* **382**, 747; 1996).

Speaking in New Delhi, Chirac suggested that an offer of this sort, which he had made in private to Inder Kumar Gujral, the Indian prime minister, would permit "new progress on the road to disarmament and non-proliferation". According to some reports, technology from France and other countries — and India's inclusion in the G8 group on nuclear safety — may be offered to entice India to change its position. This is the main obstacle to the CTBT coming into force.

Ozone monitoring over Europe stepped up

[BRUSSELS] The European Commission last week launched a campaign to monitor and study the loss of atmospheric ozone over Europe, following two earlier campaigns that had focused on ozone loss in the Arctic stratosphere. The Third European Stratospheric Experiment in Ozone, which is funded jointly by the European Union (EU) and national agencies, will run until the end of next year, and will involve more than 400 EU scientists, with participation

from Canada, Iceland, Japan, Norway, Poland, Russia, South Africa, Switzerland and the United States.

A major goal of the initiative is to gather data relevant to the long-term ozone decline over Europe, where total column ozone levels in winter and spring have already been found to be more than 10 per cent lower than those in the late 1970s.

Earthquake risk shuts down German reactor

[MUNICH] Germany's highest administrative court has given a definitive ruling against the operation of the nuclear power reactor Mülheim-Kärlich in the Rhine valley near Koblenz, because of the risk of earthquakes. The DM7 billion (US\$3.8 billion) plant was built in the early 1980s, but operated for only 13 months, between 1986 and 1988. It has been idle since a court ruled in favour of local protesters who argued that the risk of earthquakes had not been investigated sufficiently.

The operating company, RWE, which has no further right to appeal, says the reactor will cost DM1 billion to demolish. RWE intends to claim DM9 billion in compensation from the *Land* (state) of Rheinland-Pfalz, where the plant is located, on the grounds that the *Land* had initially approved the reactor in 1975.

India softens rules on foreign scientists

[NEW DELHI] The Indian government has relaxed the rules governing the export of human biological material, giving foreign biomedical researchers easier access to blood samples, DNA and human cell lines. Restrictions on work by visiting scientists using Indian material have also been removed, although they will not be able to carry out fieldwork on their own.

The new rules, published recently by the health ministry, replace controversial regulations that have been in place since 1992 prohibiting the transfer of any human material to a laboratory abroad without permission from the Indian Council of Medical Research. The only exception was transfer of blood samples or other material for diagnostic purposes for which facilities were not available in India.

Gore speaks out against genetic discrimination

[WASHINGTON] Vice-President Al Gore last week called on the US Congress to bar employers from using genetic information to discriminate in the workplace. In a speech at the National Academy of Sciences to the Genome Action Coalition, a non-profit group that supports genome research, Gore

said that genetic progress "should not become a new excuse for discrimination".

Gore argued that fear of genetic discrimination is already prompting Americans to avoid genetic tests "that could literally save their lives". He asked Congress to draft legislation barring employers from requesting or requiring genetic information before hiring new employees, and from using it to discriminate against those already employed.

Russia's researchers to be left out in the cold

[MOSCOW] Almost half of Russia's scientific organizations were recently warned that their heating systems are to be switched off because they have not paid their heating bills. That was the message delivered to Vladimir Bulgak, the Russian vice prime minister whose various responsibilities include science, at a meeting with scientific trade unions and members of the Russian Academy of Sciences.

Such a move could have disastrous consequences in the middle of winter, and would be likely to lead to strikes by researchers. But Bulgak responded by pointing out that the government had done much last year to support scientists, whose salaries had increased by about 30 per cent, to reach 87 per cent of the national average.

Callan's canyons and Voronoi's cells

Sir — A recent Art and Science article by Martin Kemp¹ describes sculptures that Jonathan Callan makes by sprinkling cement powder uniformly over a horizontal board in which small holes have been drilled at random, so that some of the powder flows out through the holes and is lost while the rest piles up to form a fantastic landscape in miniature.

Here it is noted that the structure of these sculptures may be described by the techniques of computational geometry and, intriguingly, has affinities with a recent model of the architecture of the Universe on very large scales.

Powder sprinkled on a horizontal board in which there is only one hole yields a uniform layer with a hollow cone centred on the hole, the slope of the walls of the cone being constant at the steepest slope the powder can sustain without grains in the surface slipping downhill under the pull of gravity.

The artistic and mathematical interest arises in the case in which there are so many holes that the cones intersect, leading to a sculpted landscape where the holes are separated by steep slopes terminating in razor-sharp ridges and peaks.

The key to the underlying geometry comes from noting that any grain of powder that flows out through the board does so through the hole nearest to its starting position in the layer.

It is then possible to draw on the board

the catchment region of each hole, because it consists of all the points in the plane of the board that are closer to that particular hole than to any other, which is the definition of a geometrical entity termed the Voronoi cell.

When the holes are so small that they may be regarded as Euclidean points, the Voronoi cells take the simple form of convex polygons, in which the boundary between any cell and its neighbours consists of several straight line-segments enclosing the parent hole (Fig. 1).

Each segment is the perpendicular bisector of the line joining two neighbouring holes, and the ridges in the sculpture lie directly above these line segments.

The altitude profile of the ridges is also of interest: it is the curve given by the intersection of the vertical plane through the corresponding Voronoi cell boundary with the cone on either side, and is therefore a hyperbola.

The sculpture may readily be modelled in the computer and, as may be seen from the example in Fig. 2, the results have more than a passing resemblance to the photographs of the real thing.

The Voronoi tessellation is a mathematical concept that has many applications in a range of sciences — enough to fill a thick book² — and the biggest of these applications is undoubtedly a model for the large-scale structure of the

Universe as traced by the galaxies.

It has been found that the galaxies have a foam-like distribution in which they inhabit thin sheets at the boundaries between huge voids.

The Voronoi tessellation appropriate to points scattered at random in three-dimensional space consists of irregular polyhedra, the boundary between any adjacent pair of cells being the plane that perpendicularly bisects the line joining their two points. The 'Voronoi foam' model for the structure of the Universe³ has the galaxies sited only in these boundary planes.

This resembles the way in which the powder accumulates at the boundary ridges in Callan's sculptures while avoiding the conical canyons. Although the sculptures have a typical scale of centimetres rather than hundreds of millions of light years and are derived from a two-dimensional rather than a three-dimensional Voronoi tessellation, they give a direct insight into one of the most popular models to emerge in recent years for the large-scale structure of the Universe.

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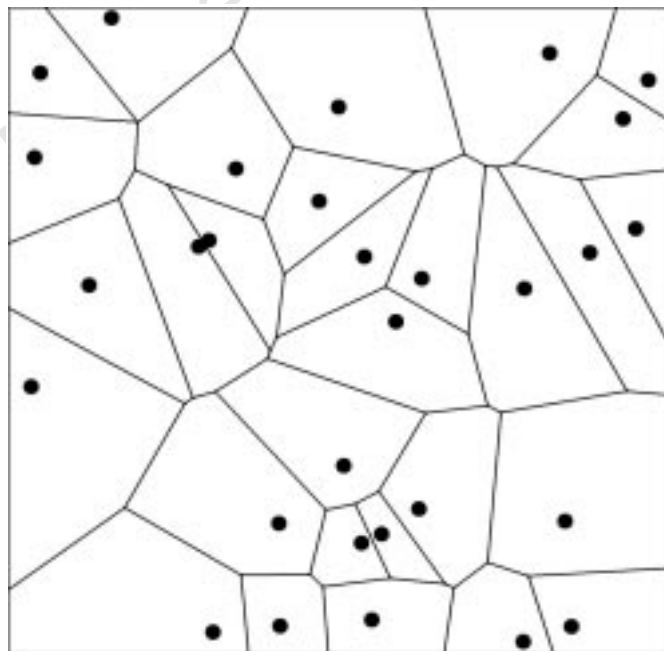


Figure 1 Voronoi cells in the plane. When holes in a board are so small that they can be regarded as Euclidean points, the cells take the form of convex polygons. Each polygon encloses all the points in the plane that are closer to the associated node (black dot) than to any other node.

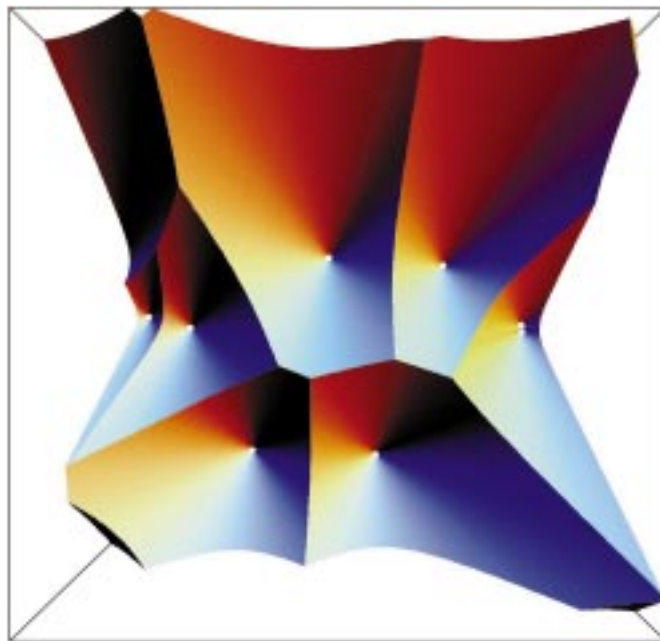


Figure 2 A computer model of one of the cement sculptures, in a perspective view from above. The surfaces depicted are those of hollow, intersecting cones with their apices sited at random in a plane. The ridges separating the apices lie directly above the straight line-segments of the Voronoi tessellation of the plane, and their altitude profiles are hyperbolae.

Maintaining science culture in Japan

Two of Japan's government agencies are set to be merged in an attempt to raise the country's scientific standards. But unless a 'bottom-up' approach to science is adopted, the culture of basic research may be damaged.

Minoru Oda



In a planned reorganization of its science-related government agencies, Japan is to merge the Ministry of Education, Science, Sports and Culture (Monbusho) with the Science and Technology Agency (STA). It is hoped that this will strengthen science in higher education and bring healthier practices and greater flexibility to the funding system of the education ministry. But there is a danger that the culture of basic research cultivated in Japanese academic institutions in the past century will be lost with the introduction of 'top-down' science administration.

The roots of this culture can be traced back to the Meiji restoration near the end of the nineteenth century, when Japan's first university, modelled on European (particularly German) lines, was established in Tokyo as the first of seven 'Imperial' universities. Although some Japanese scientists gained international repute in those early days, the overall academic level of the scientific community was far below that of the West: most scientists trained in Europe but retained the intellectual values of the old Edo Era culture.

In the early twentieth century, Japan changed from being an agricultural society to an industrial one. Its isolation from the West during the First World War led to the establishment in 1917 of the Institute of Physical and Chemical Research (RIKEN). It was set up as a semi-private organization with a donation from the Imperial family in the belief that Japan's survival depended on the promotion of basic research in science and technology in organizations run in parallel with the Imperial universities.

After the Second World War, the centre of scientific research had become the United States rather than Europe, and Japanese intellectuals were shocked to discover how far they had been left behind. Under US influence during Japan's occupation, the education system was extensively changed. The concept of the seven Imperial universities, based on classical European academic ideals, was replaced with 'democratic' and 'average' university education for the masses. Hundreds of new national and private universities raised the standards of education of 'blue-collar' workers and boosted Japanese industry. And in a backlash against the previous era of militaristic interference, academic freedom was also strengthened.

But in this rapid expansion, funding for university research was spread far too thinly, and there was a need for more focused investment. So the National Research Institutes for Joint University Use, including the Institute of Space and Astronautical Science and the National Laboratory for High Energy Physics, were established. Today's 14 member bodies are important for the cultivation and coordination of a variety of disciplines.

The Ministry of Education has come to understand that basic science is a valuable cultural asset. It has also started to listen to academics, adopting a 'bottom-up' approach to policy formation. But other science-related government ministries and agencies, such as the STA, adopt a 'top-down' approach. They view science as the basis of technology (and so industry) rather than as part of culture. That 'science' and 'technology' are separate concepts based on different philosophies and strategies is often overlooked by bureaucrats, politicians and the public.

The exception is RIKEN, now under the administration of the STA, which has retained a spirit of autonomy and flexibility. A potential benefit of the merger of the STA and Monbusho is that RIKEN could be adopted as a model for research organizations outside the universities, such as the National Research Institutes for Joint University Use, as its founders intended 80 years ago.

But there is also a danger that the 'top-down' approach of the STA could destroy the freedom and autonomy of research institutes under Monbusho. Consider for example the Institute of Space and Astronautical Science (ISAS) under Monbusho, and the National Space Development Agency (NASDA) under the STA. Politicians and bureaucrats might think it would make sense to merge these two space agencies when the STA and Monbusho merge. After all, what other country maintains two space agencies?

But here we see the contrast in their management styles. ISAS, an offshoot of the University of Tokyo, is an academic research organization devoted to astrophysics, space science and technology. It is small, but flexible

It is hoped that the STA will bring much-needed new funds to basic science. But a flexible 'bottom-up' approach will be essential

and autonomous. Space programmes are run by scientists and engineers, from proposal to implementation, including the design and production of satellites and launch vehicles, and launch and flight operations. It provides opportunities for research to the space-science community worldwide.

NASDA, on the other hand, is an applications-oriented agency that implements Japan's national space policy under the 'top-down' guidance of the STA. Its objectives reflect domestic and international policy objectives, such as establishing the infrastructure of Japan's space activity and participating in the Space Station Programme. The merger of ISAS and NASDA or the National Aerospace Laboratory might provide Japan's space programme with greater flexibility in the scale and budget of its operations, but the academic pursuit of space research could be overwhelmed by other forces with stronger and more immediate political appeal.

Indeed, according to one US astrophysicist, the idea that the merger will make Japanese space policy more efficient is too naive. He points out that many US and European researchers envy Japan's dual system and praise the accomplishments of ISAS. But they have been quick to emphasize the institute's limited budgets and facilities. For example, on visiting the Halley Mission Control Center at ISAS in 1986, someone from the US space agency NASA remarked: "we were encouraged that planetary exploration may still be possible with such limited and poor facilities". Another recent visitor to ISAS commented that the world's first space-based satellite for Very Long Baseline Interferometry developed by the institute with a large deployable antenna was like "Marilyn Monroe wearing vagabond's rags".

These statements highlight the modest support for basic science in Japan. Although the government enacted the Basic Science and Technology Law in 1996 to promote science and technology, only applications-oriented research fields have so far benefited. In fact the budgetary ceilings for the next fiscal year, which begins on 1 April, for institutes such as ISAS and the National Astronomical Observatory have been drastically lowered.

It is hoped that the merger of Monbusho with the STA, which is faring better financially under the law, will bring much-needed new funds to basic science. But a flexible 'bottom-up' approach will be essential. □

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Peeling the Chinese onion

Jared M. Diamond

There are many explanations for the technological decline of China at the end of the mediaeval period, and the coincident technological rise of Europe. One, in a word, is geography.

In mediaeval times, the region that led the world in technological innovation was China. By contrast, Europe north and west of the Alps was a backwater that had invented nothing of significance except for improved watermills. How did China lose its enormous lead in science and technology to Europe? Two papers by Graeme Lang^{1,2}, rich with broad implications, address this paradox in terms of structural or ultimate causation.

China's contrast with Europe has been the subject of much discussion³⁻⁶. The commonest interpretation — that Confucianism's conservative influence stifled science and technology in China — dissolves under scrutiny. For nearly 2,000 years under Confucianism, China reigned technologically supreme, with a long list of innovations ranging from canal lock-gates, gunpowder and magnetic compasses to paper, printing and sternpost rudders. Mediaeval Christian Europe was ideologically more hostile to scientific inquiry and innovation than was Confucian China. Attribution of China's eventual lag to its lack of Europe's Greek heritage or capitalistic outlook proves equally unconvincing. All such cultural interpretations, even if they were valid as proximate explanatory factors, would still beg the question of ultimate cause. As Lang remarks¹, "But even if culture was a factor, we are left with the problem of explaining why the cultures of these two regions were so different".

Lang begins by pointing out that the rise of scientific inquiry in Europe developed within a peculiarly European institution: autonomous universities where critical inquiry was relatively uninhibited by governmental or religious authority. Between AD 1450 and 1650, 90% of Europeans now considered to be contributors to science received university educations, and half of them held career posts at universities⁷. There was no comparable institution in China. Why not?

Historical causation is like an onion, whose concentric layers must be peeled back in sequence to reveal the ultimate causes at the centre. Lang sees the autonomous universities on the onion's outer skin as springing from an underlying layer of European political fragmentation. Mediaeval Europe was still divided into a thousand independent

statelets, whereas China was already unified in 221 BC. So it proved impossible to suppress critical thinking for long in Europe: a thinker persecuted in one statelet could (and often did) merely walk into the next. To take just one example, the astronomer Johann Kepler was always able to keep one step ahead of the authorities by moving between Tübingen, Graz, Prague, Linz and Silesia.

Technological innovations were as hard to suppress in Europe as was scientific inquiry: when some princes tried to suppress firearms, printing or ocean-going ships, inventors found support from another prince. Competition between statelets provided a positive incentive for them to adopt innovations that might yield military or economic advantages over their rivals. (One such beneficiary was Christopher Columbus, whose schemes for ocean exploration were rebuffed in five states before he received backing from the sixth, Spain.) In contrast, China's unity meant that the decision of a single emperor could block an innovation over the whole of China — the demise of China's clocks, ocean-going fleets and water-powered spinning machines being only the most flagrant instances.

Thus, at the onion's core rests this question of ultimate causation: why was political unification easy in China but impossible in

Europe? After China's initial unification in 221 BC, that unity disintegrated several times but was always restored, whereas not even the determined efforts of Augustus and Charlemagne (and later Napoleon and Hitler) could ever unify Europe. In partial explanation, Lang cites a contribution from Wittfogel's⁸ much-debated 'hydraulic hypothesis'. The potential for increasing agricultural productivity in the major river valleys of climatically dry north and central China by large-scale hydraulic engineering projects favoured the rise of centralized states there, whereas purely local control sufficed for maximal productivity of Europe's rainfall-based agriculture.

But the ultimate reason for Europe's political fragmentation emerges from a glance at a map of Europe (see Fig. 1). Seas, a highly indented coastline, high mountains and dense forests divide Europe into many peninsulas, islands and geographical regions, each of which developed political, linguistic, ethnic and cultural autonomy. Each such region became one more natural experiment in the evolution of technology and scientific inquiry, competing against other regions. Conversely, China has a much less indented coastline, no islands large enough to achieve autonomy, and less formidable internal mountain barriers. (Even China's two largest islands, Hainan and Taiwan, each has less than half the area of Ireland; neither was a major independent power until Taiwan's emergence in recent decades; and, until recently, Japan's geographical isolation kept it much more remote politically from the Asian mainland than Britain has been from mainland Europe.) China was linked from east to west by two parallel, long and navigable rivers, and was eventually linked from north to south by canals between those rivers. So once a unified Chinese state was founded, geogra-

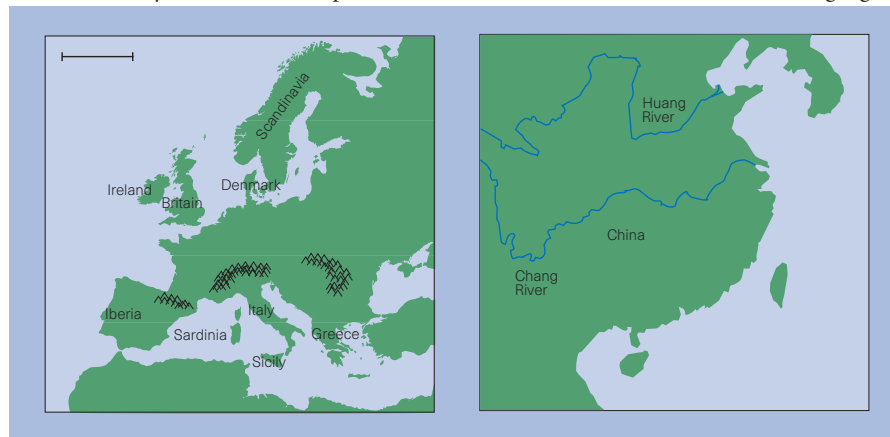


Figure 1 Sketch maps of Europe and China. Europe's coastline is much more indented and includes more peninsulas; the continent also has internal mountain chains, such as the Pyrenees, Alps and Carpathians, and two large islands. Graeme Lang argues^{1,2} that it is this comparative geographical fragmentation in Europe that resulted in the persistence of many independent states, as compared with the long-standing political unity of China. Competition between states, he proposes, permitted and fuelled the wave of scientific and technological innovation that began in Europe from the mid-fifteenth century AD. Scale bars are 500 miles.

phy prevented any other state from gaining lasting autonomy in any part of China.

Lang's analysis obviously has broader implications, of which four may be mentioned briefly. First, China's Great Proletarian Cultural Revolution of 1966–76 illustrates Santayana's famous dictum, "Those who cannot remember the past are condemned to repeat it". Just as China's unity permitted a disastrous decision by one emperor to abolish ocean shipping throughout China after AD 1433, a disastrous decision by Chairman Mao Zedong shut down the entire educational system for one billion of the world's people.

Second, the advance of technology may be hindered not only by excessive unity but also by excessive geographical fragmentation (take, for instance, New Guinea, and possibly India). Some intermediate degree of fragmentation, with moderate connectedness between the fragments, may be optimal for science and technology⁶. The problem of devising that optimal intermediate fragmentation is acute in Europe today. Current attempts to unify Europe appear to run counter to thousands of years of European history and to the source of Europe's strength. How can Europe now achieve an optimal balance between unity, easy communication, local diversity and local autonomy? If disunity has been good for Europe, might Britain equally profit from fragmentation into England, Scotland and Wales? In the business world, is organization into semi-autonomous but intercommunicating units the key to success of large corporations?

Third, political unity and also technological innovativeness fluctuate with time within the same geographical region. An analysis of Middle Eastern, Indian and Chinese history by Cosandey⁹ suggests that these two types of variation may be correlated in time as they are in space: for example, that ups and downs in China's technological progress arose from temporal fluctuations in China's political unity.

Finally, Lang's broadest message is that historians need to think more in terms of ultimate causes, and less in terms of culture as an arbitrary independent variable whose local idiosyncracies defy understanding. In his words²: "One of the advantages of this kind of account is that it escapes the circularity which often creeps into explanations which do not go deeper than social or cultural differences between Europe and China. Such explanations can always be challenged with a further question: Why were Europe and China different with regard to those social or cultural factors? Explanations rooted ultimately in geography and ecology, however, have reached bedrock."

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Remote sensing

Appreciate the gravity

Thomas A. Herring

Two satellites will soon be launched that can measure annual variations in the Earth's gravity due to mass changes equivalent to 1 cm of water over 250,000 km² — an area smaller than the Caspian Sea. This is gravity measurement of unprecedented accuracy. It will affect nearly all areas of study of the Earth, with the greatest advances expected in the study of ocean dynamics, continental water-table variations, sea-level rise, glaciology, and postglacial rebound. These possible applications were discussed at a meeting last month* and have been addressed in a National Research Council (NRC) report¹.

The first mission, CHAMP, is being developed in Germany with cooperation from the United States and France, and should be launched in 1999. CHAMP is a low-Earth orbiter whose main purpose is to study the Earth's magnetic field. However, it will be equipped with three global-positioning-system (GPS) receivers looking fore, aft and up. These receivers will be used to measure atmospheric refractivity (as GPS satellites go behind the Earth) and to refine our picture of the large-scale gravity field.

But the second mission, the Gravity Recovery and Climate Experiment (GRACE), to be launched by the US space agency NASA in 2001, is expected to provide the more detailed view of the changes in the Earth's gravity field. GRACE will be a pair of satellites, separated by a few hundred kilometres, and orbiting at an altitude of about 600 km for 3–5 years. These satellites will accurately measure changes in their separation, produced as they orbit the Earth following the bumps in its gravity field (Fig. 1). If the predicted measurement accuracy is realized, these satellites will give us a remarkably precise view of the Earth's gravity field and its fluctuation.

The gravity field provides a record of the Earth's mass distribution, and so can be used to understand the structure and dynamics needed to maintain that distribution. For the more fluid portions of the Earth, gravity measurements can be used to sense motions of mass. Calculating the mass distribution

and dynamics from the gravity field is not a straightforward problem, but, through a combination of spatial and temporal analyses, insight can be gained into the processes that control these dynamics. For example, the distribution of mass in the mantle can be used to measure the vigour of mantle convection. The reliability of these inferences depends on the accuracy, spatial resolution, temporal resolution and duration of the gravity measurements.

The size of the mass changes GRACE can see depends on a number of factors. Perhaps counter-intuitively, it will be most sensitive to spread-out changes: the larger the area over which the mass change occurs, the larger the perturbations to the satellites' orbits. Also, the longer a mass change exists the more accurately it can be measured, because of the increased averaging time.

The NRC report¹ details the sensitivity of an imagined mission similar to GRACE by expressing mass changes as equivalent thicknesses of water over regions of different sizes, and over timescales of 90 days and up. For example, the groundwater level of the High

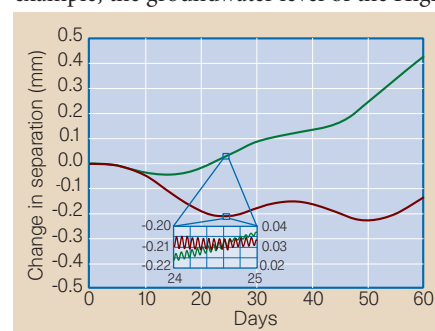


Figure 1 Sinuous grace: a simulation of the changes in separation between the two GRACE satellites, for two different mass changes on the Earth's surface. The satellites are assumed to be 400 km up in near-polar orbits, and at day zero, 1 cm of water is added over 250,000 km² at the Equator (red curve) and at 30° latitude (green). The deflections produced are much more than the expected measurement accuracy of 0.001 mm; the real problem will be subtracting the signal produced by the steady part of Earth's gravity field, a separation change of about 1 km with a periodic pattern like that in the insert.

* American Geophysical Union Fall Meeting, San Francisco, 8–12 December 1997.

Plains aquifer in the Great Plains region of the United States has fallen more than 30 m in places over the past 40 years². This aquifer covers 750,000 km². Gradual changes over regions a third of this size should be measurable to an accuracy of < 5 mm yr⁻¹ over a five-year mission; and annual variations of 10–30 mm over these same regions should also be detectable. Such integrated estimates of changes in the water volume would be hard to obtain with conventional hydrological measurements.

Over the world's ocean, sea-level changes of < 1 mm yr⁻¹ should be measurable, and, because the gravity mission will also detect the mass changes of the oceans, it will be possible to separate the thermal-expansion term of sea-level change from the water-mass term. Also, it should be possible to determine where the additional water is coming from — the current state of change of the world's large ice sheets, for example, is still poorly understood.

Interpreting changes in gravity is not without its problems. The mass distribution that generates a given external potential field is not unique. But most of the mass variations on timescales less than years are expected to arise from changes near the Earth's surface, and these can be inferred uniquely as

surface mass densities. The atmosphere must be taken into account in the interpretation of these surface density changes, because it redistributes large masses and it is the most dynamic of the fluids in and on the Earth. In many regions of the world, existing models for atmospheric mass variations will probably be adequate to remove the atmosphere's contributions — but that will not be the case everywhere.

In addition to gravity changes, the next generation of gravity missions will greatly improve our knowledge of the static portion of the field. When combined with ancillary data (seismology, geology and laboratory measurements of materials at high pressure and temperature), the static gravity field should improve our understanding of the structure and evolution of the crust and lithosphere, and the processes involved in mantle dynamics and the deep structure of the Earth. □

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Molecular neurobiology

Making memories stick?

Cori Bargmann

One goal of molecular neuroscience is to identify the molecules that mediate learning and memory, and, on page 455 of this issue, Grotewiel *et al.*¹ describe a potential player in memory formation. The identification of this molecule — a member of the integrin family of cell-adhesion receptors — expands the study of learning from neurotransmission and signalling pathways within the neuron, to include the neuronal surface where adhesion molecules collaborate to mould the connections between cells.

The fruitfly *Drosophila melanogaster* learns to avoid odours that are paired with an electric shock using a region of the brain called the mushroom bodies. When the mushroom bodies are defective or absent, flies show normal sensitivity to odours and electric shock, but they fail to learn the association between the two stimuli². Moreover, a number of genes implicated in olfactory learning are preferentially expressed in the mushroom bodies. These include several genes that are important for signalling through the second messenger cyclic AMP: *dunce*, which is a cAMP phosphodiesterase; *rutabaga*, a calcium-calmodulin-dependent adenylyl cyclase; and the catalytic subunit of the cAMP-dependent protein kinase A (ref. 3).

Cyclic AMP pathways are required in

learning, and hyperactivation of cAMP-mediated transcriptional pathways can increase the formation and retention of long-term memories⁴. These observations have led to the model that local, regulated

changes in levels of cAMP modulate the strength of mushroom-body synaptic connections during learning. But the short-term target of cAMP signalling — a hypothetical molecule that actually strengthens the synapses — has remained elusive.

Direct screens for fruitflies that fail to learn are very labour-intensive, so Grotewiel *et al.* devised a shorter path to finding new genes. They first identified 100 enhancer-trap transposon-insertion lines whose expression was enriched in the mushroom bodies. They then screened those flies for defective learning, assuming that the transposon would affect the activity of the adjacent gene. One mutant had a defect in the early phase of learning, and they called this *Volado* (*Vol*) — meaning 'absent-minded'. *Vol* mutants learn the olfactory-avoidance task only about half as well as wild-type flies. They show normal olfactory acuity and normal responses to shock, indicating that their learning defect is not an indirect result of poor perception or movement.

The cloning of *Vol* revealed that it encodes a molecule that had not previously been implicated in learning — a hitherto unknown α -integrin subunit. The protein is expressed on the axons and dendrites of the mushroom-body neurons, including their synaptic regions (Fig. 1). Integrins are transmembrane receptor proteins that bind extracellular-matrix and transmembrane proteins⁵. Different cell types express many integrin types with different ligand-binding specificities that can participate in cell-matrix attachment, cell-cell adhesion and cell signalling. The initial *Vol* mutation was identified based on its learning defects, but stronger mutations are lethal, indicating that the gene has essential functions in other tissues. ▶

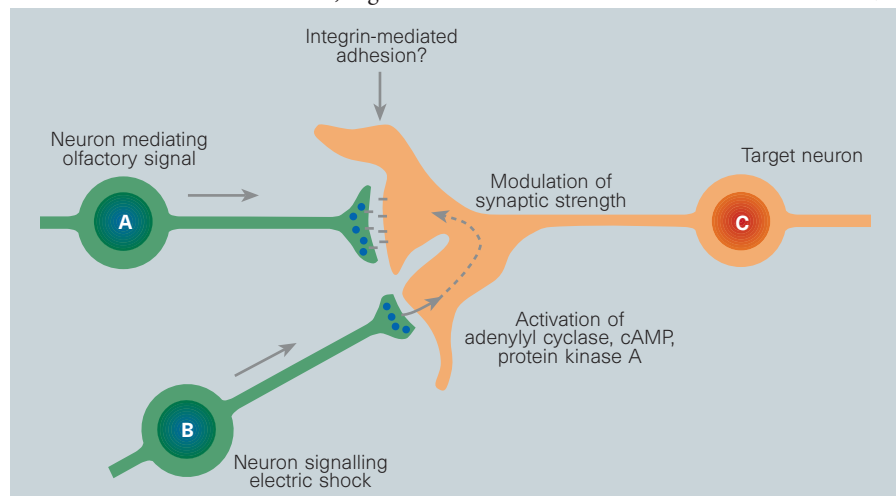


Figure 1 One model for memory formation in the mushroom bodies. If neuron A (olfactory input) and neuron B (electrical shock input) are simultaneously activated, the cyclic AMP/protein kinase A pathway is engaged, and integrin-mediated adhesion between A and C is modulated. The neurons that mediate these responses and the sites of action of these genes are unknown. Grotewiel *et al.*¹ have identified a *Drosophila* protein called *Volado*, which belongs to the integrin family of cell-adhesion receptors. *Volado* is required for memory formation, although the mechanism through which it acts is not known.



100 YEARS AGO

'A Diagram of Heredity' – Francis Galton. The law of heredity which was formulated by myself ... and which, as I am exceedingly gratified to learn, is now strongly corroborated by an independent investigation, has recently been illustrated by a useful diagram. ... The two parents between them contribute on the average one half of each inherited faculty, each of them contributing one quarter of it. The four grandparents contribute between them one quarter, or each of them one sixteenth; and so on, the sum of the series $1/2 + 1/4 + 1/8 + 1/16$ &c. being equal to 1, as it should be. ... The area of the square diagram represents the total heritage of any particular form or faculty that is bequeathed to any particular individual. It is divided into subsidiary squares, each bearing distinctive numbers, which severally refer to different ancestors. The size of these subsidiary squares shows the average proportion of the total heritage derived from the corresponding ancestors. ... The Subject of the pedigree is numbered 1. Thenceforward whatever be the distinctive number of an ancestor, which we will call n , the number of its sire is $2n$, and that of its dam is $2n + 1$.



All male numbers in the pedigree are therefore even, and all female numbers are odd.

From *Nature* 27 January 1898.

50 YEARS AGO

During a television outside broadcast from Brands Hatch, in Kent, during the afternoon of August 31, 1947, there appeared on the screens and from the loudspeakers of two television receivers ... a sudden and intense increase of fluctuation noise. The visual effect was that of a violent snowstorm of the type well known to viewers due to a motor-car ignition interference, but at a very much more intense level than seen heretofore by either of the observers. ... It would seem beyond reasonable doubt that the interference observed was, in fact, solar noise, particularly when the radio of television band-width (5,000 kc./s.) to recording receiver band-width (about 20 kc./s.) is taken into account. From *Nature* 31 January 1948.

The learning defect of *Vol* mutants resembles that caused by destruction of the mushroom bodies. Is *Vol* simply a protein required for the function of this brain region, or is it more central to learning? In one very nice experiment, Grotewiel *et al.* introduced a wild-type copy of the *Vol* gene into *Vol* mutants under the control of a heat-shock promoter. Inducible expression of *Vol* protein by heat shock three hours before the learning assay was enough to rescue learning in the mutants. As the *Vol* protein disappeared in the hours following heat shock, the ability to learn new olfactory tasks diminished as well. So *Vol* activity is required right at the time of learning — not during the initial development of the mushroom bodies. Furthermore, although they seem to represent a loss of gene function, *Vol* mutations are dominant, indicating that learning is sensitive to levels of *Vol*. The gene encodes two alternative transcripts, and mutations that disrupt either one are sufficient to give a dominant learning defect. This unusual genetic dosage-sensitivity has been observed for other important learning genes such as the cAMP mutants³, and it strengthens the argument that *Vol* participates directly in learning.

Exactly how *Vol* acts in learning is less obvious. The *Vol* integrin could convey a signal to the mushroom-body neurons that acts in parallel with the cAMP signal or modulates its production. Or *Vol* could maintain certain interactions between neurons. Unfortunately, it is not possible to monitor the activity of *Drosophila* mushroom-body neurons but, because other learning mutants affect synaptic function at more easily studied synapses⁶, the same might be true of *Vol*.

One interesting possibility is that *Vol*-mediated adhesion contributes to the strength of synapses between two cells, and that this adhesion is dynamically regulated by learning (perhaps by cAMP signalling itself).

Integrin function can be regulated by intracellular and extracellular signals. For example, lymphocyte integrins are inactive until they receive inflammatory signals (from tissue) that engage their adhesive properties and allow invasion of the blood vessel⁷. Other cell-adhesion molecules such as *Aplysia* APCAM and *Drosophila* fasciclin II are downregulated by signals that alter the strength of synapses^{8,9}. Perhaps such adhesion modulation is a more general property of neuronal plasticity. If so, these results raise the interesting (but still very speculative) possibility that learning and memory involve rapid changes in the physical properties of synaptic connections — a literal tightening or loosening of the association between two neurons. □

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DNA recognition

Reading the minor groove

Claude Hélène

The design of molecules that recognize specific sequences on the DNA double helix would provide new tools to control gene expression and a rational basis for fresh approaches to drug development. Parts of each base pair are exposed in the two distinct 'grooves' of DNA, the major and the minor grooves, and the sequence information of the DNA duplex is available for read-out from both of them. On page 468 of this issue¹, White *et al.* describe a molecular code for the recognition of the four Watson–Crick base pairs from the minor groove of DNA using hairpin polyamides containing imidazole, pyrrole and 3-hydroxypyrrole rings.

Double-helical DNA can bind different types of ligand which can be classified in two categories: intercalators which insert their aromatic ring between two adjacent base pairs, and groove binders which bind DNA

within either groove of the double helix. Intercalators have a limited sequence specificity, because they are interacting only with two base pairs unless they are linked to a groove-recognition element — as exemplified by natural molecules, such as actinomycin; or by synthetic conjugates where an intercalator has been covalently tethered to a major groove ligand, such as an oligonucleotide², or to a minor groove ligand such as distamycin³. In living organisms, the reading of sequence information on DNA involves binding of proteins to specific control regions of the genes. These proteins exploit all three modes of binding: intercalation or partial insertion of aromatic amino acids, binding of α -helices into the major groove, and binding of loops of polypeptide chains or β -sheets into the minor groove. But no general amino-acid/base-pair code is available yet.

As Fig. 1 shows, it should be possible to distinguish the four base pairs (G•C, C•G, A•T and T•A) from the major-groove side through hydrogen-bonding interactions. Oligonucleotides provide a partial solution to this problem; they recognize oligopurine•oligopyrimidine sequences of double-helical DNA by forming triple-helical complexes^{4,5}. But it remains a challenge to design nucleoside analogues that would allow oligonucleotides to recognize all four base pairs (and not only two of them)⁶.

Studies in the mid-1980s, on A•T-specific minor-groove ligands, suggested that it should be possible to design ligands that could recognize G•C base pairs by replacing the pyrrole (Py) rings of distamycin or netropsin by imidazole (Im) rings (the so-called lexitropsins)^{7,8}. But this strategy had limited success in recognizing extended sequences. The breakthrough came with the discovery that 2:1 complexes could form with two distamycin molecules bound side-by-side to the minor groove of A+T-rich sequences⁹ — leading to the idea that covalent linkage of two such monomeric ligands would constitute a strong ligand for the minor groove at A+T-rich sequences^{10,11}. Indeed, hairpin polyamides with Py/Py 'pairs' could recognize A•T and T•A base pairs¹². Introducing imidazole instead of pyrrole rings provided a recognition element for guanine, with Im/Py pairs recognizing G•C and Py/Im recognizing C•G base pairs (the replacement of the C(3)H of pyrrole by N(3) allows for the formation of a hydrogen bond with the NH₂ group of guanine)^{13–16}. The degeneracy of the recognition code for A•T and T•A remained, however.

Based on the observation of an asymmetrically placed cleft on the minor groove surface at A•T base pairs, White *et al.*¹ now report that replacing the C(3)H group of pyrrole by a bulkier C(3)OH group (in 3-hydroxypyrrole; Hp) enables hairpin polyamides to recognize all four base pairs with the following molecular code: Py/Im → C•G; Im/Py → G•C; Hp/Py → T•A; and Py/Hp → A•T. The A•T/T•A discrimination is, however, obtained at the expense of stability, but destabilization is higher when 3-hydroxypyrrole is on the A side rather than the T side. In the example described by White *et al.* (see Fig. 2), replacing the Py/Py pair of 1 by a Py/Hp pair as in 2 decreases binding to the sequence 5'-TGGTCA-3' 191-fold and that to 5'-TGGACA-3' by sixfold only. Conversely, when the Py/Py pair is replaced by a Hp/Py pair as in 3, the destabilization is reversed with 5'-TGGACA-3' destabilized about 250-fold and 5'-TGGTCA-3' only sixfold. This result illustrates a common phenomenon in molecular recognition: specificity may be achieved by increased destabilization of interactions at unselected sequences, rather than by enhancing interactions at the specific sites.

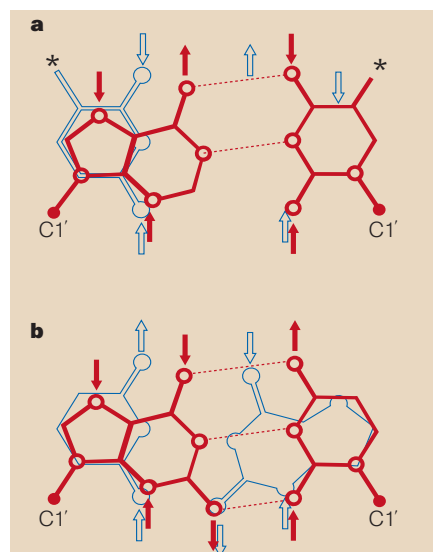


Figure 1 Differences in DNA base-pair recognition between the major and minor grooves. **a**, A•T superimposed on T•A and **b**, G•C superimposed on C•G, so that the C1' atoms of the glycosidic linkages coincide. The arrows indicate the positions where hydrogen bonds may form (but not their direction), and point to the O and N hydrogen-bond acceptors and away from the NH₂ hydrogen-bond donors. The hydrogen-bonding positions in the minor groove (lower edge of the base pairs) nearly coincide when the base pairs are reversed. In contrast, the hydrogen-bond donor and acceptor groups in the major groove (upper edge), as well as the methyl group of thymine (*), occupy different positions following base pair reversal. Major-groove ligands should be much better for discriminating between the four base pairs, but White *et al.*¹ show that minor groove ligands can be designed successfully to achieve this end. (Adapted from ref. 16.)

White and colleagues' results do not provide evidence for the exact molecular basis of the observed discrimination. It would be surprising if the OH group of 3-hydroxypyrrole plays only a steric role. Hydrogen-bonding interactions might lead to different distortions at A•T and T•A base pairs. Replacement of H by OH at the C(3) position of pyrrole may also change the hydration of the hairpin polyamide, and differential hydrophobic effects might be involved in discrimination of A•T from T•A. The effect might also depend on the neighbouring base pairs. The substrate described by White *et al.* (GAC versus GTC) has G•C and C•G neighbours. The A•T/T•A discrimination is reported to be observed when neighbours are also A•T base pairs, but the extent of discrimination might vary with the environment and the local polymorphism of DNA.

To achieve selective recognition of a single gene in human cells, a sequence of about 17 base pairs must be recognized⁶. This might be difficult with hairpin polyamides — the repeat distance between two consecutive units does not exactly match

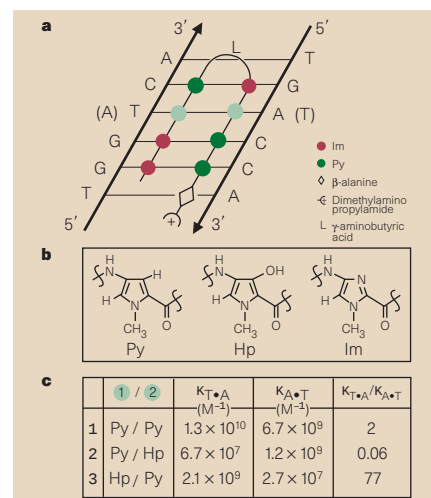


Figure 2 Hairpin polyamides. **a**, Hairpin polyamide binding to the minor groove of B-DNA, the helix being unrolled so that the base pairs are horizontal and the helix axis vertical. **b**, Chemical structures of the N-methyl derivatives of pyrrole (Py), imidazole (Im) and 3-hydroxypyrrole (Hp) used by White *et al.* to build the hairpin polyamides. **c**, Binding constants for the hairpin polyamides with pyrrole and/or 3-hydroxypyrrole rings at positions ① and ② and the two sequences where a T•A base pair is replaced by an A•T base pair. The last column shows the ratio of the two binding constants for each of the ①/② combinations.

that between two consecutive base pairs, and flexible spacers have to be used to restore the register of the recognition elements¹⁷. But might recognition of fewer base pairs allow for gene-specific effects? Within the cell nucleus, DNA sequences are partly buried because of the nucleosomal organization of chromatin and the large number of proteins bound to the genetic material. If so, shorter recognition sequences might suffice, as most identical sequences might not be accessible. If the target sequences on the DNA double-helix are within gene regulatory regions then, given that they are accessible to regulatory proteins, the same should apply to ligands such as polyamides or oligonucleotides. However, other sequences within the transcribed regions are also accessible, as shown for 15-mer oligonucleotides recognizing the major groove of DNA¹⁸. Further studies are required to tackle these issues.

Finally, we know little about the behaviour of hairpin polyamides in a cellular environment. Hairpin polyamides can inhibit gene-specific transcription in cell cultures at micromolar concentrations even though they bind naked DNA at subnanomolar concentrations¹⁹. We have no data yet on their uptake and compartmentalization in cells, and we don't know whether they bind to cellular components other than nucleic acids. All of these questions must be investigated for hairpin polyamides and related molecules before their promise as gene-

specific control agents *in vivo* is clearly established. □

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Genome sequencing

Genes blossom from a weed

Joseph R. Ecker

The tiny weed *Arabidopsis thaliana* (Fig. 1) has received much attention lately, not only from plant scientists (who are already familiar with its attributes), but from genome sequencers, federal granting agencies and even the US Congress¹. Why is this so? With its small genome² of around 120 million base pairs (Mb), its compact growth and the ease with which it can be genetically manipulated, *Arabidopsis* serves as a model for physiological, biochemical, cell biological and developmental studies of over 250,000 species of plant.

On page 485 of this issue, Bevan *et al.*³ describe their analysis of just under 1.9 Mb of contiguous DNA sequence produced by the European Union *Arabidopsis* Genome Project. Unlike the larger genomes of its distant cousins — soybean, corn, wheat and most other agriculturally important crop plants — the *Arabidopsis* genome is chock-full of

genes. On average, the authors found one gene every 4,800 bases and, by extrapolation from this gene density, they predict that the maximum number of genes needed to 'grow' a plant is about 21,000. This number is in line with estimates based on complementary DNA sequencing programmes^{4,5}.

Although this sequence represents only about 1.5% of the *Arabidopsis* genome, it provides a 'nuts and bolts' view of the largest contiguous segment of plant DNA sequenced to date. To put things in perspective, it is longer than most of the completely sequenced prokaryote genomes. Like previous reports of functional cataloguing⁶ and whole-genome annotation, Bevan and colleagues' snapshot of predicted *Arabidopsis* genes (and the stuff in between) will probably be out of date soon after publication. However, their careful analysis of this 1.87-Mb sequence provides a tantalizing preview of the bricks and mortar needed to build a plant.

Searches of sequence databases with the 389 predicted *Arabidopsis* genes revealed that bits and pieces of just over half (some 209 genes) can be recognized in the genomes

of organisms ranging from the bacterium *Escherichia coli* to humans. Because of the unique nature of plants, the authors had to add several additional categories/sub-categories to the functional catalogue⁶ of genes. These included genes involved in the production of secondary metabolites, the source material for numerous pharmaceutical products. Moreover, because plants can't up and run from their predators, a category was established for genes involved in disease resistance, defence and responses to a variety of stresses.

Plant species diverged relatively recently, so, when complete, this catalogue of gene sequences will allow *Arabidopsis* to serve as a reference genome or 'gene bank' for all flowering plants. But the flip side of the coin reveals that it may not be easy to assign a function to 46% of the genes. Extrapolating from the data, nearly 10,000 genes in the *Arabidopsis* genome will fall into the category of 'function unknown', providing plant biologists with fertile ground for new investigation, and with a big challenge — to assign functions to these genes.

These estimates are supported by the analysis of a more broadly based collection of largely non-contiguous sequences produced by the *Arabidopsis* Genome Initiative^{7,8} (Fig. 2). This international consortium of genome sequencers was established in 1996 as part of the Multinational Coordinated *Arabidopsis* Genome Research Project — a model for international cooperation in science. Bevan *et al.*³ are involved in this project, as well as genome researchers in France, Japan and the United States. By mid-1998, the US, Japanese and EU groups are expected to deliver around 30 Mb of sequence (about one-quarter of the genome), distributed across the five *Arabidopsis* chromosomes (Fig. 2). These groups will soon be joined by researchers at a genome centre in France, which is just coming on-line.

The current rate of sequencing by the *Arabidopsis* Genome Initiative is on target with the agreed date of 2004 for completion of the genome sequence. However, this year Congress has provided additional funding to the National Science Foundation (NSF), for the establishment of a Plant Genome Research Program and a scaling up of the US effort. These extra funds will allow the target date to be brought forward. In a recent announcement by the NSF, the planned scale-up of the NSF/Department of Energy/US Department of Agriculture Interagency *Arabidopsis* Genome Sequencing Program calls for the genome sequence to be finished by the end of the year 2000. Given this boost in funding — and pending continued support for the other members of the *Arabidopsis* Genome Initiative — the international community of plant scientists should be prepared for a bountiful harvest of genes at the dawn of the new millennium. □



Figure 1 *Arabidopsis thaliana* — a little weed, but a powerful model. A tiny-seeded member of the mustard family, *Arabidopsis* is the model for over 250,000 species of plant.

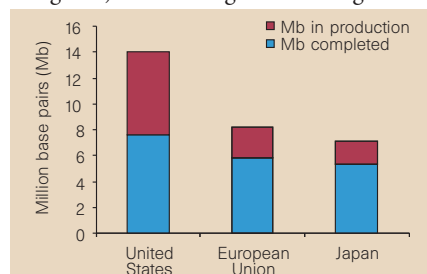


Figure 2 Progress in the *Arabidopsis* Genome Initiative. As of December 1997, 18.90 million base pairs (Mb) of the *Arabidopsis* genome had been sequenced by the *Arabidopsis* Genome Initiative, 1.9 Mb of which are described by Bevan *et al.*³. Another 10.69 Mb of sequence is in production and should be completed by the middle of this year.

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Atomic physics

Positronic lithium and the many-body problem

J.-P. Connerade

At one level, physics is simply about interactions between particles. In a sense, then, it fails miserably, because if more than two particles or bodies are involved, no general solution can be found to their equations of motion — only partial solutions for special cases. The many-body problem troubled such celebrated scientists as Newton and Poincaré, and as the world we live in is dominated by many-body interactions, it is a highly relevant problem. A surprising new tool to explore the many-body problem has been found in atomic physics: G. G. Ryzhikh and J. Mitroy, of the Northern Territory University, Australia, have shown¹ that it should be possible to attach a positron to a neutral lithium atom. Why is this useful? To explain that, a brief excursion into chaos and quantum mechanics is required.

By considering the three-body problem, Poincaré grasped the significance of chaos in classical mechanics — that the behaviour of even simple systems can be exponentially sensitive to initial conditions, making it impossible to predict beyond a certain timescale. In other words, although all classical many-body systems are deterministic, many have no tractable solutions.

In quantum physics, the many-body problem emerges again. But true chaos is excluded from quantum mechanics because of the Uncertainty Principle: the very existence of chaos requires infinite divisibility of space, which only occurs in classical physics. At first sight, this is very promising — one might hope to make better progress with the many-body problem in quantum physics. Also, the Pauli exclusion principle, which tells us how to construct a wavefunction for several identical particles, seems designed to assist. In reality, the situation is much more complicated: the many-body problem persists at the quantum level, and the role of chaos remains mysterious². Although useful approximations are found, the problem is fundamentally unsolved.

There are several approaches to solving it. Many physicists take the view that the 'best' systems in which to study many-body effects are those where the forces between the constituents are completely known. From this standpoint, atoms are ideal. The force

involved is the Coulomb force, arguably the best documented in the whole of physics. But a complexity of quantum systems stems from the Pauli principle. When several electrons are present, one must include not only the direct interaction between them, but also a quantum-mechanical force — the exchange interaction — whose origin is the indistinguishability of particles.

Fortunately, nature provides a tool to probe this exchange force. This tool consists in substituting a positron for an electron. Because these are distinguishable, the exchange force disappears. Other differences emerge, as the charge of the positron is also of opposite sign, but many-body theories can be tested more thoroughly in this way than just by comparing theory with experiment for a given atom.

In principle, there are two ways for a positron to cling to an atom. A positron and an electron together form bound states of a 'pseudo-atom' called positronium (Ps). This is neutral, but one can attach it to an atom, forming a new kind of molecule. Several such molecules are stable. For example, positronium hydride (PsH) was predicted, and has been observed³. This is less surprising than seems: removing the positron leaves behind a negatively charged ion, so one can understand how the positron becomes bound.

The second is less obvious. One can also attach a charged particle to a neutral system, provided the latter possesses an electric dipole moment of its own, or can be distorted (polarized), in which case a dipole is induced by the particle that attaches to it. Atoms do not possess an intrinsic dipole moment, so the second mechanism must act. Negative ions occur for many atoms, of course — even positronium forms a negative ion⁴.

But can one attach a positron to the neutral atom, to produce an exotic positive ion (a positronic ion, to distinguish it from normal positive ions)? It could then be compared with the corresponding negative ion. This strategy depends on whether a bound state exists for any atom, which is by no means obvious. All depends on whether the induced dipole is sufficient to bind a charged

particle; that is, on whether the polarizability of the original system is large enough. That depends on many-body interactions, so it is of great interest to enquire whether a neutral atom is able to form negative or positronic ions when bombarded by electrons or positrons respectively. This question can be addressed either experimentally or theoretically.

In general, as one removes electrons from an atom to form the more usual positive ions, screening of the Coulomb force between the nucleus and the external electrons is reduced, and the central force experienced by the electrons is strengthened in comparison with the many-body forces between the electrons. We say that the many-body forces, or correlations, are reduced. On the other hand, the correlations in a neutral system or (even better) an ion produced by attaching an additional charged particle are much larger. For this reason, such ions are among the most suitable objects in which to study many-body effects.

Already, the suggestion had been made on the basis of theoretical estimates that magnesium, zinc, cadmium and mercury should possess positronic ions. However, these elements have closed outer subshells, which are less extended in space than the outer shells of the corresponding alkali elements, and therefore less polarizable.

The latest step is more decisive. The new theoretical paper¹ describes a fully *ab initio* calculation showing that lithium, the lightest of the alkalis, can bind a positron, to form a stable positronic ion. The binding energy is 0.059 eV. To discover anything new about many-body interactions in this system, actual positronic lithium will have to be produced and its spectrum measured.

More exhaustive tests could be carried out if we could also compare ions in other systems. Unfortunately, it is not yet clear how to extend the new theory to incorporate the additional closed shells of heavier alkali elements. Another route might be to make much larger spherical objects⁵ — spherical metallic clusters. By virtue of their size, clusters are even more readily polarized than atoms, and can also form negative ions⁶. Various models have been developed to represent them, and are in general similar to self-consistent field theories of the atom. So there seems to be no reason why positronic ions of metallic clusters should not eventually form a new test-bed for our theories of many-body physics. □

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Apoptosis

Death cycle and Swiss army knives

Michael O. Hengartner

Cytochrome *c* leads a double life. When a cell is called on to commit apoptotic suicide, cytochrome *c* relocates from the mitochondria to the cytosol. There, it helps to activate the foot-soldiers of apoptosis — the death proteases known as caspases¹. How cytochrome *c* escapes from the mitochondria is still a matter of debate¹, but it is clear that certain elements within the apoptotic regulatory hierarchy do not condone such behaviour. In particular, overexpression of the cell-death suppressors Bcl-2 and Bcl-xL prevents the release of cytochrome *c*, suggesting that these proteins act upstream of cytochrome *c* in the pathway to cell death^{2,3}. However, on pages 449 and 496 of this issue, Zhivotovsky *et al.*⁴ and Rossé *et al.*⁵ show that Bcl-2 can also protect cells downstream of cytochrome *c* release, forcing a re-evaluation of this newly acquired dogma.

Caspases do the brunt of the work in apoptosis. Activated through proteolytic processing, they cleave a limited number of apoptotic substrates and cause, directly or indirectly, most of the changes that are characteristic of apoptosis (for example, see ref. 6). So how are caspases activated? In the nematode worm *Caenorhabditis elegans*, the caspase homologue CED-3 is activated through a physical interaction between proCED-3 and CED-4. Interaction with CED-4 seems to be the only mechanism available for CED-3 activation, because there is no programmed cell death in *ced-4* mutant animals. In cells that should survive, CED-9 (which belongs to the Bcl-2 family) binds to CED-4 and holds it in an inactive conformation, thereby preventing CED-4-mediated activation of proCED-3. The ability of CED-9, CED-4 and CED-3 to exist in a multiprotein complex has led to the 'apoptosome' model of cell-death regulation (reviewed in ref. 7).

A series of elegant papers by Wang and colleagues indicated that at least some caspases are activated through a similar mechanism in mammals. This group purified, from human cell extracts, three proteins that can activate procaspase-3 in the presence of dATP. The molecular nature of two of these apoptotic protease activating factors — or Apafs — made perfect sense: Apaf-3 turned out to be caspase-9, which has a similar structure to CED-3 (ref. 8); and Apaf-1 was the long-sought mammalian homologue of CED-4 (ref. 9). As expected from sequence similarities with the worm proteins, Apaf-1 binds to Apaf-3/caspase-9 and promotes its proteolytic activation.

But there are two interesting twists to this

story. First, unlike CED-4, Apaf-1 needs a cofactor in order to bind to and activate caspase-9. To general stupefaction, this cofactor — Apaf-2 — turned out to be the humble cytochrome *c*, which is normally present on the outer surface of the inner mitochondrial membrane. Here, it usually shuttles electrons between systems III and IV of the mitochondrial electron-transport chain.

The second twist concerns the mechanism of cell-death suppression by the Bcl-2 oncoprotein (and its homologues). Bcl-2 interacts with at least half a dozen proteins (not counting other Bcl-2-family members¹⁰), but it is still not clear how it prevents death. Although it would be attractive to use the *C. elegans* data to postulate that Bcl-2 will bind to Apaf-1 and/or other adaptors of its ilk, evidence for such a model is woefully lacking. Rather, last year two groups^{11,12} showed that high levels of Bcl-2 can prevent the release of cytochrome *c* by mitochondria, providing an attractive biological — if not molecular — function for the protein (Fig. 1a). Could Bcl-2, prodded perhaps by the recruitment in mammals of a new apoptotic cofactor, have adopted a new function? Has the *C. elegans* model reached the limits of its usefulness?

Maybe not. The studies of Rossé *et al.*⁵ and Zhivotovsky *et al.*⁴ now suggest a more complicated picture. Using two different approaches, these groups find that high levels of Bcl-2 can delay death, even when cytochrome *c* is already in the cytosol. Rossé

et al. used transient transfection of Bax, a pro-apoptotic member of the Bcl-2 family, to bring about cytochrome-*c* release and apoptosis. Co-transfection of Bcl-2 prevented Bax-induced apoptosis, yet cytochrome *c* nonetheless transferred to the cytosol. So, at least in this case, Bax seems to act upstream of cytochrome *c*, which in turn acts upstream of Bcl-2. Zhivotovsky *et al.* used a more direct, but also more invasive, approach — they microinjected high concentrations of cytochrome *c* into the cytosol. They found that high levels of Bcl-2 block most of the deaths, indicating that Bcl-2 can act downstream of cytochrome *c*.

According to the *C. elegans* model of CED-9/CED-4/CED-3, this is exactly what we might have expected. But how do we explain the finding that the same protein can block death at two distinct steps in the apoptotic pathway? One possibility is that Bcl-2 is just very resourceful, and simultaneously performs many functions (the Swiss army knife model¹⁰; Fig. 1a). Or maybe the two activities are linked. For example, Bcl-2 has been reported to undergo a large-scale conformational change that can lead to its insertion into membranes and, possibly, the formation of Bcl-2 channels¹⁰. Such an insertion could allow the release of cytochrome *c* (by hampering the formation of Bcl-2/Bax heterodimers?), and prevent Bcl-2 from binding to Apaf-1. If not all of the Bcl-2 molecules insert, then the remaining ones could still bind Apaf-1 and prevent cell death. This might be particularly apparent if the apoptotic pathway is stimulated downstream of the events that usually lead Bcl-2 to insert into the membrane.

Alternatively, Bcl-2 might seem to act both up- and downstream of cytochrome-*c*

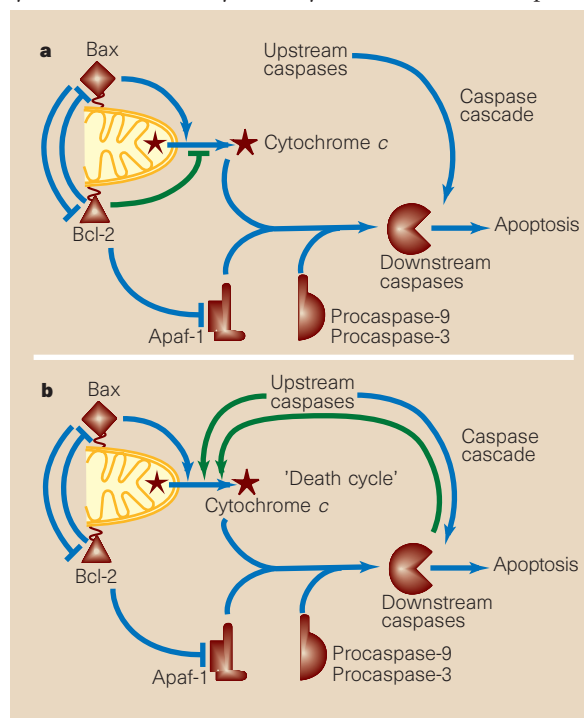


Figure 1 Models for the function of Bcl-2 in apoptosis. Based on the genetic similarity with CED-9 from *C. elegans*, both models incorporate the idea that Bcl-2 can prevent apoptosis by interacting with caspase activators. **a**, The 'Swiss army knife' model¹⁰. Bcl-2 prevents the release of cytochrome *c* (perhaps by antagonizing Bax), as well as caspase activation in the presence of cytosolic cytochrome *c*. **b**, The 'death cycle' model. Bcl-2 blocks caspase activation, preventing amplification of pro-apoptotic signals. Signals that generate high levels of active caspases might be able to bypass the amplification step and activate the downstream caspases through a direct caspase cascade.

release because it is involved in a circular pathway. Indeed, not only does cytochrome *c* stimulate caspase activation, but active caspases promote the release of cytochrome *c* from intact mitochondria. This suggests that mitochondria carry a caspase substrate that, when cleaved, promotes cytochrome-*c* release. Thus, mitochondria might act as apoptotic amplifiers, fostering a positive-feedback loop between cytochrome-*c* release and caspase activation (Fig. 1b). Any event that primes the loop — cytochrome-*c* release, caspase activation or otherwise — will initiate the vicious 'circle of death', eventually leading to large-scale caspase activation and apoptotic death.

For the death-cycle model to be viable, we need to add dampeners to the system. Otherwise, the slightest perturbation could be amplified, leading to unwarranted death. Good candidates are the inhibitors of apoptosis (IAP) family of proteins, which specifically inhibit particular caspases¹³. Bcl-2 could also be considered as a dampener, its role being to break the cycle — if the *C. elegans* model is right — by preventing caspase activation. If the cycle is broken early on, as might be the case with weak signals, there will be little release of cytochrome *c*, and Bcl-2 will seem to have acted upstream of cytochrome *c*.

In theory, Bcl-2 should also prevent strong pro-apoptotic signals, as long as they enter the cycle at the level of cytochrome-*c* release. But it might be a pyrrhic victory —

such a cell might be technically alive, but if its mitochondria are mangled and its electron-transport chain disrupted, it is unlikely to thrive or divide. Neither Rossé *et al.*⁵ nor Zhivotovsky *et al.*⁴ report on the long-term prospects for the cells with cytosolic cytochrome *c*, leaving this question open.

At this point, both of the models can account for most of the published observations. Unfortunately, the proliferation of arrows (Fig. 1) makes falsification difficult, thereby limiting the predictive value of the models. In fact, probably the only safe prediction is that apoptosis will be a very complex process or, worse, a nonlinear one. Or both. □

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evolution as the long-range attractive signal.

Mutations in the *robo* gene were originally recovered in a screen for abnormal axon-projection patterns in the *Drosophila* CNS⁹. In *robo* mutant embryos, axons meander back and forth across the midline. Mutations in *sax3* — a closely related *Caenorhabditis elegans* gene — result in a similar phenotype². The worm ventral nerve cord is asymmetrical but, nevertheless, it is divided into discrete left and right axon bundles. Axons mainly cross from left to right only at the anterior and posterior ends of the nerve cord. In *sax3* mutants, however, axons cross the midline in either direction along the length of the nerve cord. Kidd *et al.* and Zallen *et al.* have also found that the *robo* and *sax3* genes encode immunoglobulin-type transmembrane receptors, which are expressed on neuronal growth cones as they encounter the midline^{1,2}. One of the rat *robo* genes is also expressed by spinal-cord neurons when their axons are responding to guidance cues from the floor plate. So rat *robo*, like the invertebrate *robo* genes, may be reading a signal that prevents these axons from repeatedly crossing the midline¹.

A combination of a long-range attractant and short-range repellent expressed by the same midline cells would elegantly explain why most axons first project towards the midline, and, on reaching it, turn to follow a parallel pathway, never straying across the midline or back towards the periphery. But why do most axons cross the midline before making this turn? And why do they cross only once? Conceivably, crossing and non-crossing axons might differ in their responses to the attractive or the repulsive cue.

It is not yet clear whether netrins are also involved in the crossing decision, because it is experimentally difficult to separate guidance towards and across the midline. For example, the partial loss of commissures in *netrin* mutant fly embryos^{7,8} may occur because many commissural axons fail even to make contact with midline cells. Still, netrins are not the only attractive signals provided by the midline and floor plate, and commissural and longitudinal axons may differ in their sensitivity to some of these other cues.

Differential sensitivity to the midline repellent, at least in flies, is dramatically shown by Kidd *et al.*¹. The Robo protein is expressed on the growth cones of the longitudinal axons, yet it is almost completely absent from commissural axons — until they have crossed the midline. This explains why commissural (but not longitudinal) axons can cross the midline, and also why, having crossed once, commissural axons never turn back and cross again.

In a companion paper, Kidd *et al.*³ show that Robo is downregulated by a transmembrane protein encoded by the *commisureless* (*comm*) gene. As the name implies, axons

Axon guidance

A Roundabout way of avoiding the midline

Barry Dickson

Bilaterally symmetrical animals must be able to integrate sensory inputs and coordinate motor control on both sides of the body. Thus, many neurons in the central nervous system (CNS) project their axons to the opposite side of the body, whereas others project axons that remain on the same side. In the latest issues of *Cell*^{1,2} and *Neuron*³, the groups of Corey Goodman, Guy Tear, Marc Tessier-Lavigne and Cori Bargmann report that, from worms and flies to rats and humans, a common mechanism determines which axons cross the midline and which do not.

In insects and vertebrates, the two symmetrical halves of the CNS are separated by a specialized group of cells. Located at the ventral midline of the CNS, these are called midline cells in insects, and they form the floor plate in vertebrates. Most CNS axons initially grow towards these midline cells and then turn longitudinally — either on their own side (ipsilateral), or by first crossing the midline and then turning (contralateral). As they

project longitudinally, these axons skirt along the edge of the midline, but never cross it again. Those axons that cross the midline form the commissures that allow sensory information and motor instructions to pass from one side of the animal to the other.

A few years ago, there was much excitement when midline cells in worms, flies and vertebrates were all found to secrete the same type of chemoattractants, netrins, to guide axons along the initial part of their trajectories towards the midline^{4–8}. But evidence has been accumulating from studies in the fly⁹, grasshopper¹⁰, fish¹¹ and chick¹² suggesting that the midline is not only the source of a long-range attractive signal, but also of a short-range repulsive signal. Kidd *et al.*¹ and Zallen *et al.*² now identify a family of putative receptors for such a repulsive signal — the Roundabout (Robo) family. So far, one *robo* gene has been identified in worms, two in flies, two in the rat and two in humans, suggesting that the short-range repulsive signal has been just as highly conserved during the course of

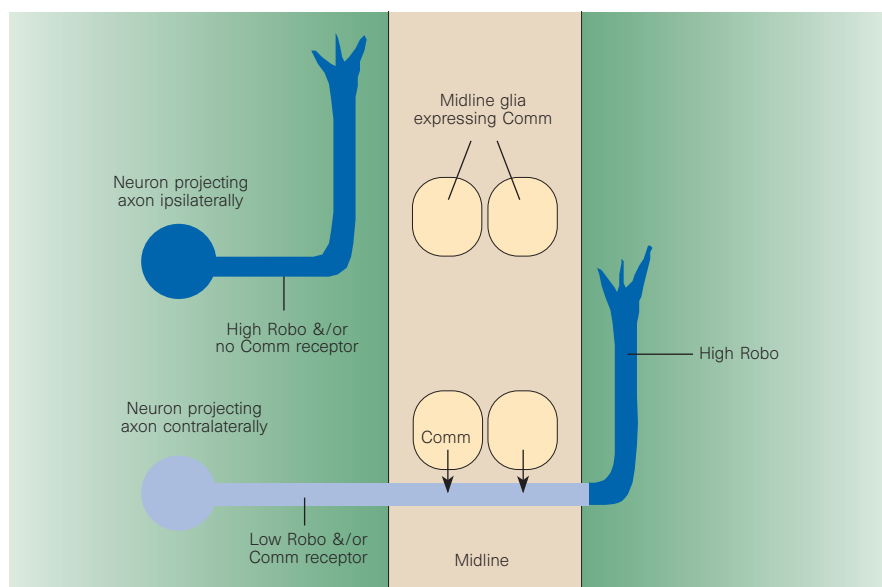


Figure 1 Model for regulation of midline crossing by axons in the central nervous system of *Drosophila* embryos. The midline is the source of a diffusible attractant (netrins; green) and, as described by Kidd *et al.*¹ and Zallen *et al.*², a membrane-bound repellent (the Roundabout or Robo ligand; brown). Axons projecting ipsilaterally (on the side from which they originated) express high levels of Robo (blue), whereas contralaterally projecting axons (on the opposite side) express high levels of Robo only after crossing the midline. The commissureless (Comm) protein (yellow) is transferred from midline glia to commissural axons¹³, where Kidd *et al.*³ have found that it downregulates Robo and thus allows these axons to cross the midline.

cannot cross the midline in *comm* mutants⁹. The Comm protein is expressed by a group of midline glial cells, positioned exactly where the commissures are pioneered¹³. The protein is transferred to the commissural axons that contact these cells, inhibiting Robo and facilitating passage of these axons across the midline^{3,13} (Fig. 1). Because both commissural and longitudinal axons contact these cells, they must differ in their ability to respond to Comm. Kidd *et al.*³ suggest that commissural axons may initially express lower levels of Robo, so they will be more sensitive to downregulation by Comm. Or, alternatively, perhaps only commissural axons express a Comm receptor.

The high structural and functional conservation in the *netrin* and *robo* families reveals a common underlying logic in the control of axonal traffic by specialized midline structures in the nervous systems of worms, flies, mice and humans — a long-range attractant guides axons to the midline, and a short-range repellent prevents them from crossing it. Commissural axons are granted temporary immunity against this repellent, allowing them just a single pass across the midline.

These studies^{1–3} raise many questions. For example, what is the nature of the Robo ligand, the putative midline repellent? How does Comm downregulate Robo, and how is Robo upregulated after the midline crossing? It will also be interesting to find out whether other species use Comm homologues to allow transit across a repulsive midline. Although no vertebrate homologue has yet

been reported, a similar mechanism probably conducts commissural axons across the vertebrate floor plate. But as sequencing of the *C. elegans* genome nears completion, it seems that worms lack a *comm* gene, just as their nerve cord lacks commissures.

Evolution seems to have enjoyed a relatively free rein in designing the CNS, coming up with different cellular architectures and developmental strategies in nematodes, arthropods and chordates. One common feature is the organization of axon projections into an orthogonal grid of circumferential and longitudinal nerve fibres. Highly conserved guidance mechanisms employing members of the *netrin* and *robo* families can account for much of this pattern. □

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Daedalus

Keep out the cold

One theory of human evolution is that our ancestors were semi-aquatic apes. They spent so much time in water that they lost their body hair, which impedes swimming. Daedalus points out that in fact, water is a deadly environment for human beings — not by drowning, but by chilling. Our alleged aquatic ancestors should have grown even thicker, longer fur to minimize heat transfer. Indeed, in a maritime accident, it is worth putting on all the clothes you can find; you will live that much longer in the water. As for swimming — forget it. It stirs away all the muscular heat it generates.

Sadly, many sea disasters happen so suddenly that there is no time to look for spare clothes. So Daedalus is devising a nautical uniform which reacts with water to form an ideal survival garment. His first inspiration was the absorptive 'super slurper' acrylate polymer used in bandages and babies' nappies. It can take up hundreds of times its weight of water, expanding into a flabby gel as it does so. In fibrous form, it can be woven into cloth. Underclothes of this fabric would swell in water into a splendid convection-proof wet-suit. But Daedalus's survival suit will not merely insulate; it will actively generate heat. He recalls the immersion batteries on aircraft life-jackets, which use sea water as their electrolyte, and power a signal lamp. His new garment will be one large distributed battery, triggered by immersion in water.

Its electrochemistry is an interesting challenge. At first Daedalus wanted it to generate hydrogen — perhaps enough of it to fill a balloon and lift the wearer out of the water. But more sanely, he now wants it to exploit the high energy of metal oxidation. A distributed zinc–air battery, exploiting the oxygen dissolved in the water, seems best. A few hundred grams of zinc could keep the wearer warm for hours in the coldest water. Hydrogen generated in a side reaction might usefully inflate buoyancy pockets in the garment.

Swollen by gas and absorbed water, the survival suit will usefully discourage attempts to swim. Its wearer may generate a little added heat by shivering, though this also will stir away all the metabolic heat thus mobilized. Only young babies can combat cold by passive thermogenesis. Advocates of our aquatic origins are welcome to the threadbare argument that their ability is a vestigial remnant of our ancestral watery metabolism.

David Jones

Obituary

David N. Schramm (1945–97)

Astrophysicist who linked outer space to the inner space of particle physics

David N. Schramm thought big. Twenty-five years ago, few shared his optimism about cosmology, whereas today most cosmologists speak of a golden age. The standard hot Big-Bang model already accurately describes the Universe from the time it was 0.01 seconds old; and the future is also promising, with bold ideas prompted by fundamental physics now being tested by an avalanche of data. No one appreciated this confluence of theory and observation more, and very few, if any, did more to bring it about than David Schramm, who died on 19 December when his aeroplane crashed near Denver.

As a graduate student at Caltech, Schramm worked with William A. Fowler and Gerald Wasserburg. Having two advisors, each of great stature, was an early indication of the energy Schramm would show throughout his career. Both advisors were part theorist and part experimentalist, and pioneers in applying nuclear physics to astrophysics in order to understand the origin of the chemical elements in stars and elsewhere. David would take it one step further, applying elementary-particle physics to evolution of the Universe. He began his career studying the origin of the heaviest elements in the explosions of stars (supernovae), but his most important work involved the production of the lightest elements — deuterium, ^3He , ^4He and ^7Li — in the Big Bang.

Big-Bang nucleosynthesis was first studied in the 1940s, but it was David Schramm who realized its potential as an extraordinary probe of cosmology and fundamental physics. In the process, he helped to create the field of 'particle physics and cosmology'.

Schramm took a special interest in deuterium, using it as a 'baryometer', to deduce the mass density of baryons (neutrons and protons) present in the Universe today. In 1972, Schramm and his colleagues reasoned that the present deuterium abundance could be used to set an upper limit on the density of baryons: 10% of the critical density necessary to halt the expansion of the Universe. Any denser than that, and too little deuterium would have been created in the early Universe.



The implications are profound. Measurements of the dynamics of galaxies and clusters of galaxies tell us that the total amount of matter is at least 20% of the critical density, and perhaps much more — so deuterium clinches the argument for the existence of some form of exotic dark matter, not made of familiar baryons. The leading candidate is slow-moving elementary particles left over from the earliest moments, called cold dark matter.

Another light isotope, ^4He , allowed Schramm to make an extraordinary contribution to fundamental physics. The amount of ^4He made in the Big Bang depends upon the number of types of neutrino, increasing with additional neutrino species. As there is one neutrino species for each type of electron and pair of quarks, the species number determines how many fundamental particles there are altogether.

In 1977 Schramm, Gary Steigman and James Gunn used observations of the present helium abundance to limit the possible number of neutrino species to fewer than seven. At the time, only two neutrino species were known, but it was believed that there might be many more, and laboratory experiments could only limit the number to be less than around 5,000. Over the next ten years, refinements in theory and in the observations led to a cosmological limit of three species. Finally, in 1989 experiments at high-energy accelerators confirmed that there are indeed three species.

Schramm also encouraged his colleagues to explore the flip side of this 'inner-space/outer-space' connection: the implications that particle physics has for astrophysics. Today, the leading explanation for the decades-old solar-neutrino problem is neutrino oscillation — an hypothesized fundamental property of neutrinos. And in cosmology, the leading ideas to extend the hot Big-Bang model, namely inflation and the cold-dark-

matter model, are motivated by elementary-particle physics.

Schramm helped establish particle astrophysics and cosmology as a vital area of research. In 1982, he and Leon Lederman, the director of Fermilab, convinced the US space agency NASA to fund a theoretical astrophysics group at a particle-accelerator laboratory. His own contributions were multiplied by those of the 24 thesis students he supervised and the even greater number of postdocs he mentored at the University of Chicago, as well as the countless young scientists whose careers he influenced around the world.

Schramm was a most effective spokesman for cosmology, astrophysics and basic research, reaching the general public as well as world and national leaders. He was also a physicist of the highest energy. In the month after his death, for example, he was scheduled to attend a scientific conference in Aspen; the World Economic Forum in Davos, Switzerland; a Council meeting of the American Astronomical Society in Washington; a PhD defence in Paris; and a Physics Division Advisory Committee meeting at Los Alamos.

Schramm's zest for life was as big as his enthusiasm for science. Affectionately known as Big Dave, in his collegiate days he was a champion Greco-Roman wrestler, and he continued his involvement in wrestling as a coach at Caltech and Chicago. He was an avid mountaineer, climbing in Colorado, South America, the Caucasus, the Alps and the Tatra. Although he turned down an opportunity to climb Everest, Dave proudly proclaimed that by climbing Chimborazo in Ecuador he had reached the farthest point from the centre of the Earth (owing to the equatorial bulge). And as both of us can testify, he was a game competitor on the racquetball court, basketball court and ski slope.

Dave had a passion for flying, and over the years owned four aeroplanes. But of his primary passion there was never a doubt: his red Porsche bore the licence plate "BIG BANG", and he was the President and Chief Pilot of "Big-Bang Aviation".

Because of his energy and enthusiasm, many of his colleagues described Big Dave as a *primaeval* force of nature. We can only add that he was a most positive force, and a force that will be dearly missed.

Edward W. Kolb and Michael S. Turner

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Abbott's absolutes

Berenice Abbott was fascinated by the challenge of capturing on film motion too rapid for the human eye. Her scientific photography has given a new meaning to the term 'portraiture'.

Martin Kemp

The 'objective eye' of the camera and the impersonal traces of light on a photographic emulsion were hailed as powerful tools in scientific recording virtually from the public announcements of the new medium in France and England in 1839. In that very year William Henry Fox Talbot wrote to the astronomer and physicist Sir John Herschel that he had "great hopes" for photographs taken with his "Solar Microscope... as for instance in copying the minute forms of crystallisation which are so complicated as almost to defy the pencil".

Not the least of the potentialities of the artificial eye has been the capturing of motion too rapid for human sight, most famously as in the revelations of a horse's gait by Eadweard Muybridge in 1878. No one has explored these potentialities more potently than the American photographer Berenice Abbott (1898–1991).

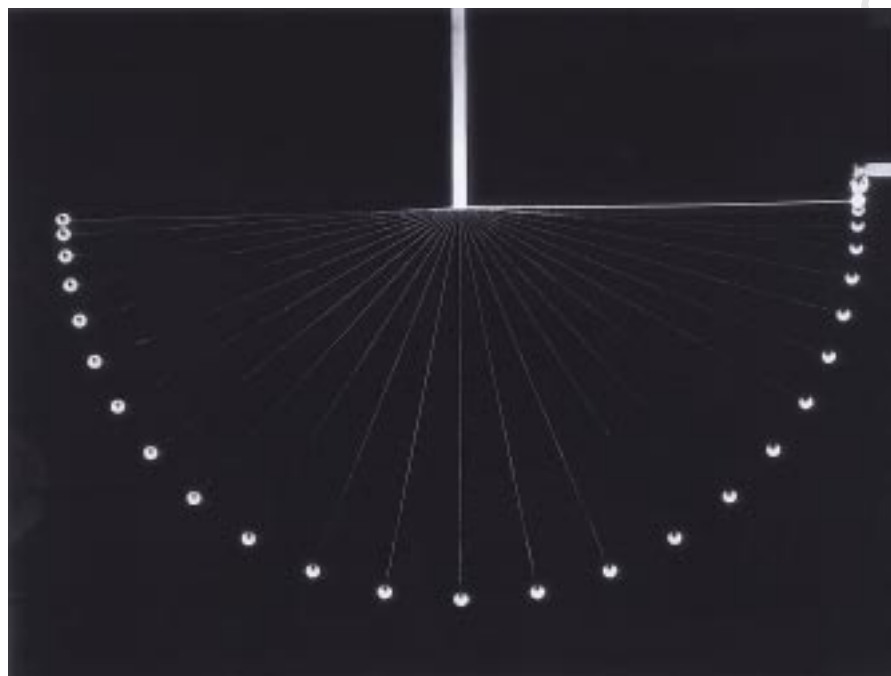
In the 1930s Abbott was associated with a group of 'objectivist' photographers who looked to the philosopher Alfred North Whitehead's *Science and the Modern World* (1925) and *Process and Reality* (1929) in their attempt to define a remorseless form of impersonal realism which revealed the 'absolute' as manifested in the appearance of things.

The tone of the endeavour is conveyed by her remarks in 1929 on Eugène Atget, the devoted photographer of a depeopled Paris: "As an artist, he saw abstractly, and I believe he succeeded in making us see what he saw." Best known for her portraits and cityscapes, Abbott's most innovative work involved the reform of the techniques, communicative values and aesthetics of the photography of scientific phenomena.

She served as photographic editor of *Science Illustrated* and collaborated on two high-school science books, *Physics* and *Physics: Laboratory Guide*. Using a projection system she christened 'Supersight', she at first concentrated on the kinds of traditional subjects that directly revealed their structure to the lens, such as insects' wings and soap bubbles.

During the 1940s and 1950s she invented devices that could capture invisible motions, not least the behaviour of waves and the paths of fast-moving bodies, which were so engaging contemporary physicists.

She was particularly drawn to some of the classic problems of Galilean dynamics, such



Abbott's *Transformation of Energy*, 1958–60 (top) and *Wave Interference Pattern*, 1950s.

as the path of a projectile fired upwards from a moving platform and the response of a pendulum to gravitation. Her exploitation of high-speed flash and dark backgrounds allowed the phenomena to 'draw' their own diagrams in a way that surpassed in vividness and beauty any conventional diagrammatic representation.

Her aim was neither to act as a mere documenter of phenomena nor to exploit scientific photography for its decorative potential. Rather, she sought to use those properties of photography as a visual medium that distinguished it from painting

and hand-made graphics to speak eloquently of the transformation of energy in terms of invariable structures embedded in phenomena.

The spirit of her endeavour is nicely captured by the heading to a two-page spread about her work in the *New York Times Magazine* in 1959, "Portraits of Natural Laws". Like any good portraitist, Abbott knew that a good likeness makes huge technical and aesthetic demands on the artist. □

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Oldest known ant fossils discovered

Here we report fossil ants, including a new genus of Ponerinae, about 50 million years (Myr) older than the previous oldest specimens. These discoveries in amber from the Turonian stage (92 Myr ago) of New Jersey in the United States have important implications for estimates dating the origin of ants, and extend the age of an extant ant subfamily back about 50 Myr.

Until now, a specimen of *Sphecomyrma freyi* in a piece of amber from Cliffwood Beach, New Jersey, was one of the few non-compression fossil ants from the Cretaceous period showing most morphological details¹. The metapleural gland is the only morphological trait unique within the Hymenoptera that distinguishes ants, and can be seen in most ants embedded in amber. This gland produces antibiotic-like substances², necessary to maintain nests underground or in humid pieces of wood, where bacteria and fungi would otherwise invade immobile broods. The gland is seen in modern species, opening above the hind coxae. Development of the gland and eusociality were probably correlated and are involved in the great ecological success of the ants. In an Amazonian rainforest, for example, ants make up more than 25% of the total animal biomass³.

Wilson *et al.*¹ described an almost perfectly preserved worker of *S. freyi*, but the debate remained whether this fossil is an ant because the presence of the metapleural gland was uncertain. *Sphecomyrma* was thus excluded from a recent phylogenetic analysis of the Formicidae⁴, and its relatively short first antennal segment in workers was assumed to prevent the basic social behaviour of trophallaxis⁵.

Our new specimens include three worker and four male ants. One complete, well-preserved worker was the second discovery of *S. freyi*. Among other well-preserved traits in this specimen are the external and even part of the internal anatomy of the metapleural gland. The gland has a circular opening and a very large subcuticular atrium (Fig. 1a). *Sphecomyrma* is thus an ant.

Two of the males are a new species of the Cretaceous genus *Baikuris*, known previously only from Upper Cretaceous (Santonian) amber from Taymyr, northern Siberia. The

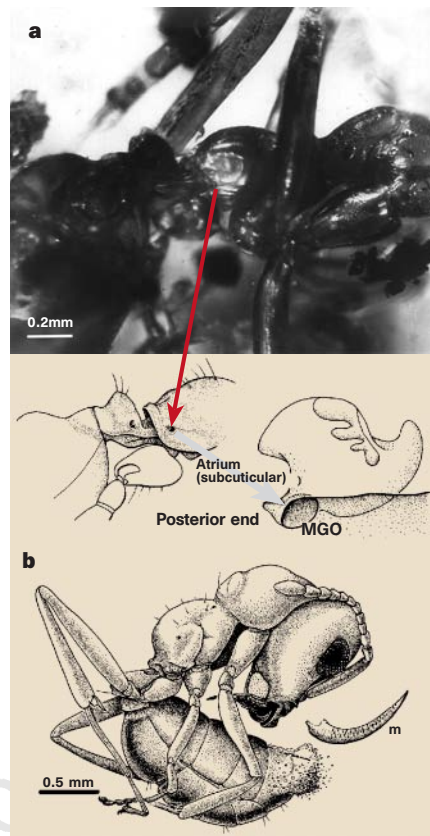


Figure 1 Photomicrographs of New Jersey ant fossils. **a**, Alitrunk and portion of the gaster of the new *S. freyi* worker with a drawing showing the position and structure of its metapleural gland (MGO). **b**, Lateral view of the new Cretaceous ponerine genus (m, mandible in frontal view). A detailed description will be provided elsewhere⁶ (http://research.amnh.org/entomology/social_insects).

third male belongs to a genus as yet undetermined. The fourth male is tentatively assigned to *Sphecomyrma* and would be the first known male of that genus.

Another worker represents a new genus, distinguished by its clubbed antennae, proportions of the antennal articles, thin mandibles that lack teeth and cross extensively when closed, and a girdling constriction of the gaster typical of the large modern subfamily, the Ponerinae (Fig. 1b). Furthermore, the broad attachment of the third and fourth

abdominal segments, and the apical denticles on the genae, indicate a relationship with ants of the ponerine tribe Amblyoponini.

Addition of these two new taxa confirms the basal position of *Sphecomyrma*⁶, but as part of a quadritomy. The position of the new ponerine within one clade of the Ponerinae demonstrates that one major lineage of extant ants was established well before the formation of amber from Sakhalin Island (probably Palaeocene)⁷ and of Eocene Baltic amber⁸, which are about 50 Myr younger than the New Jersey amber.

A reasonable estimate would thus place the origin of the ants into the lowermost Cretaceous (about 130 Myr ago), but probably no older. This conclusion is consistent with the relationship of the Formicidae to the wasp families Vespidae and Scolidae⁹ and the phylogenetic position of the Cretaceous Vespidae¹⁰. On this basis, a hypothesis of a Lower Jurassic origin of ants¹¹, based on estimated divergence time of mitochondrial cytochrome *b* sequences, is unlikely.

Although ants originated in the Cretaceous, it was not until the Tertiary⁷ period that they became so dominant and diverse in terrestrial ecosystems, as documented in Baltic⁸ and Dominican amber¹². We are still examining why the radiations were delayed until the Tertiary.

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Climate change and Australian wheat yield

Nicholls¹ reported that 30–50% of the increase in Australian wheat yields in the period 1952–92 resulted from climate change. He estimated a simple linear rela-

tionship where a 1 °C fall in diurnal temperature range increased Australian wheat yield by 0.52 t ha⁻¹. This effect, taken with the trend in diurnal range, accounted for 45% of the yield increase between 1952 and 1992. In an extended model with three climate variables, Nicholls found that changes in minimum temperature had had most impact on wheat yield and that rainfall change had con-

tributed little. In our view, Nicholls's results need qualification and should be interpreted with caution. They are only estimates, and do not include standard errors to indicate their precision.

The analytical challenge is to separate the effects of climate change from other factors affecting Australian wheat yields. Non-climate factors include total wheat area, the

location of Australia's wheat plantings, previous soil cropping history, management, technology and the levels of conventional inputs. Nicholls did not use data on non-climate factors, which he proxied by a linear trend in yields. Simple statistical procedures for removing trend effects include adding a trend variable to relationships between series, or working with first differences of the series. The procedures are not equivalent. The choice is not arbitrary but relates to whether the trends are deterministic or stochastic.

Nicholls did consider deterministic climate trends, the extent of which, together with the estimated effects of climate change on yield, provided his estimates of the contribution of climate change to wheat yield increases. But he 'detrended' using first differences, an approach more appropriate to stochastic trends. Differencing is consistent with deterministic trends if a yield-trend effect is included as an intercept in the relationship between the first differences of yields and climate variables. Unless yields have a random-walk component, the error term in the relationship between first differences would be correlated with the first differences of the climate variables, as the errors include a model-misspecification component. Ordinary least-squares estimates of the difference relationship would thus be biased. Nicholls both forced the intercept to be zero and used ordinary least-squares.

Improvements in wheat varieties and farming systems from 1952 to 1992 enabled wheat producers to cope better with weather conditions by use of larger machinery, 'knockdown' herbicides, increased and better fertilizers, changes in crop rotations, and more drought-tolerant varieties. Location of wheat production also moved significantly towards 'new', drier, more 'open' land. Yield responses to non-climate factors are probably non-linear. They are also likely to depend on climate, and vice versa. Such interactions should be recognized by including interaction variables in yield models. When omitted, such effects must become part of the error term, be picked up by the trend effect, or become part of the estimated climate effect. As interaction variables tend to correlate with climate changes, their omission would probably bias the estimated climate effects.

Even without interactions, estimated climate effects might be biased. Nicholls noted the possibility of confounding climate and non-climate effects where these factors are correlated, and that farmers might alter inputs in response to seasonal conditions. Farmers can adjust fertilizer applications, timing and extent of plantings, pesticide use, and both management and harvest effort. First differences of climate variables are then likely to be correlated with variations in these inputs, and estimated climate effects con-

founded with the effects of these inputs. In Nicholls's data, yield might be better modelled by diurnal range, trend and a diurnal range-trend interaction than without the interaction term. Yield change is thus a function of variables other than change in diurnal range.

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We believe that Nicholls's conclusion¹ — that reduced frost frequency has been predominantly responsible for 30–50% of the 45% increase in average Australian wheat yield between 1952 and 1992 — is not correct.

Nicholls correlated the year-to-year differences in average wheat yield from the cropping zones, which comprise less than 10% of the Australian land area, with year-to-year differences in annual precipitation, and maximum and minimum temperatures averaged across the continental area. Wheat is grown only over the winter/spring period within a small fraction of that climatic range. Additionally, since 1952 the range of wheat production has extended substantially northwards in eastern Australia and expanded greatly in Western Australia.

Assuming that the correlations between year-to-year differences in national average yield and weather variables would apply also to decadal changes, Nicholls attributed almost half of the increase in national average yield over four decades to a reduction in diurnal temperature range. Further analysis of partial correlation coefficients attributed no causation to rainfall and emphasized a strong underlying relationship with average minimum temperature. Low availability of soil water, however, might still explain low yields if a correlation exists between minimum temperature and low rainfall in Australian wheat-growing zones.

Rimmington and Nicholls² have shown low correlations between the Southern Oscillation Index and wheat yield in southern Australian states but better correlations for Queensland, New South Wales and Western Australia, as well as an overall significant correlation for the continent, which these authors attributed to rainfall. Thus, by explaining reduced wheat yields by decreased frost incidence, Nicholls might have incorrectly attributed causality to correlation. Drought years in the wheat districts tend to be less cloudy and have lower minimum temperatures.

We think that a correlation of yield with annually averaged minimum temperature is unlikely to be closely related to frost effects. Yield is damaged by frost only during the flowering period of a few days. Australian cereal growers have traditionally been very concerned about frosts, preferring late-flow-

ering varieties and planting dates that give near-certain frost-avoidance, thereby losing yield in most years through end-of-season drought. Thus it is unlikely that the scale of yield loss from frost 40–50 years ago was sufficient to give scope for a 10–20% increase through less frost damage.

The 10-year period from 1983, when the national average yield was higher than it had been over the level trend-line of the previous 30 years (see Fig. 1 of ref. 1), was characterized not only by warming but also by new technologies. These included the almost complete adoption of high-yielding semi-dwarf wheat varieties, greater use of nitrogen fertilizer, better crop rotations (especially in areas where yields have increased most, capitalizing on earlier planting opportunities made possible by new herbicide and tillage practices), the adoption of winter varieties enabling earlier planting, and the use of earlier-flowering wheats that specifically shifted the balance towards more frost risk and less drought effect. Although it is conceivable that decreasing spring frost frequency for northeast Australia³ has helped the latter strategy, we think it could not have been directly responsible for as much as 10–20% of the yield difference before and after 1982.

The national rainfall increase from 1952 to 1992 of 39 mm (ref. 4) could be agriculturally significant. If, for example, it applied to the wheat areas and fell entirely during the growing season it could account for the yield increase observed. But an analysis of rainfall trends in wheat-growing areas since 1952 (ref. 4) reveals a slight decrease in growing season rainfall, rather than an increase. National and, more importantly, regional correlations between rainfall and wheat yield¹ show clearly that the analysis needs to be made more specific to the wheat-growing season and wheat regions. It has also been shown⁵ that the observed yield increases have not occurred uniformly but patchily in already high-yielding regions, and despite yield decreases in some areas producing lower yields.

A multiple regression of absolute yield on climatic variables is more straightforward than the 'first-difference' approach of Nicholls¹ because the correlation between yields of consecutive years seems to be small whereas there is an inherently stronger correlation between consecutive first-differences. Hence it is more difficult to specify reliability for the estimates of the effects of climate change.

The first-difference approach also biases against influences with low short-term variability relative to long-term change such as change in atmospheric CO₂ concentration. Unlike inter-annual differences in temperature and rainfall, the effects on yield of the small monotonic annual increase in CO₂ concentration are cumulative, but their

impact cannot be discerned by the first-difference method.

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Nicholls replies — Godden *et al.* state that I proxied non-climate factors “by a linear trend in yields”. I did not and my conclusions were not dependent on the functional form of any relationship between non-climate factors and yield. They suggest that forcing the intercept to zero and fitting with ordinary least-squares biases my estimates. I repeated the analysis without forcing a zero intercept, with no significant effect on the results. The climate–yield change relationship is so strong (see Fig. 2 of Ref. 1) that other forms of fitting produce a result very similar to ordinary least-squares. This relationship dominates the effects of other variables and provides a way to estimate the effect of climate on yield change. Godden *et al.* believe that detrending with first differences is inappropriate. As I noted, repeating the analysis on residuals from fitted non-linear trends produces very similar results.

Gifford *et al.* suggest that the temperature–yield relationship simply reflects the effect of rainfall on yield. Although rainfall was related to yield and temperature, the temperature–yield relationship was stronger. Even if the temperature–yield relationship partly reflects the influence of rainfall, there is a direct temperature effect as well. They believe the correlation between minimum temperatures and yield is unlikely to reflect the effect of frost damage. Yet there is a substantial literature showing concern about the effects of frost on Australian wheat yield.

Gifford *et al.* propose the use of multiple regression of absolute yield on climate variables. Many other factors affect yields. These would confound the use of such a regression of absolute yield on climate variables. Detrending, either through first-differencing or calculating residuals from fitted non-linear trends (I did both), is necessary to avoid this confounding. They suggest that regression on absolute yields would avoid underestimating the impact of the atmospheric CO₂. I did not need to, and did not, estimate the CO₂ impact on yield, but did check that the increasing CO₂ had not confounded my analysis. It had not.

Godden *et al.* restate my comment that farmers varying their inputs in response to climate might lead to an overestimate of the effect of climate. For instance, in a year with poor yield the farmer might decide not to harvest. Next year, with good climate condi-

tions and good yield, the farmer harvests. The observed increase in yield, from one year to the next, could be attributed to increased harvest effort. However, the ultimate cause of the increased yield is the better climate, even if this is mediated partly by farmers’ responses.

The comments of Godden *et al.* and Gifford *et al.* do not negate the conclusion that climate trends seem to have led to increased Australian wheat yield. Their comments indicate the difficulties in calculating the precision of the estimated yield increase owing to climate trends. Because of these difficulties, I did not attempt to calculate the precision of the estimate.

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Injected cytochrome *c* induces apoptosis

Mitochondria, the cell’s energy-producing organelles, are thought to play a central role in mediating apoptosis, or programmed cell death. Mitochondrial morphology remains intact during the process, the apoptosis-blocking protein Bcl-2 is localized in the outer mitochondrial membrane¹, several

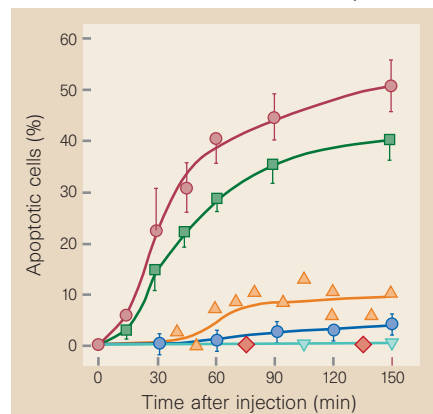


Figure 1 Effect of cytochrome *c* microinjection in NRK cells. Wild-type cells were injected with 20 μM (purple) or 10 μM (green) cytochrome *c*, with vehicle alone (turquoise) or with 20 μM biotinylated cytochrome *c* (red). Wild-type cells were preincubated with Z-VAD-fmk (30 min) and injected with 20 μM cytochrome *c* (blue). Cells overexpressing Bcl-2 were injected with 20 μM cytochrome *c* (orange). Each point represents the mean of at least four experiments involving injection of at least 50–100 cells.

critical steps in the process require ATP², and mitochondrial ‘megachannels’ or ‘permeability transitions’ open during apoptosis. It has been suggested that mitochondrial constituents leak out through these permeability transition channels, and activate the apoptotic machinery in the cytosol³. Here we show that the injection into cells of a key mitochondrial protein, cytochrome *c*, activates apoptosis.

Recently, three apoptotic protease activating factors (Apaf-1–3) were purified using a cell-free system based on cytosol from normally growing cells. One of these, Apaf-2, was identified as cytochrome *c*. This suggests that mitochondria may release cytochrome *c* during apoptosis⁴. Conversely, Bcl-2 has an inhibitory role in this translocation of cytochrome *c*, and prevents the activation of the cytosolic caspases and apoptosis^{5,6}.

We used a microinjection technique to load different cell types with cytochrome *c*. Types tested included adrenocortical Y-1 tumour cells, normal rat kidney (NRK) epithelial cells, mouse embryonic Swiss 3T3 fibroblasts and rat promyelocytic IPC-81 leukaemia cells. In all these systems, cytochrome *c* was able to kill cells by apoptosis in a dose-dependent manner (Figs 1, 2b), death occurring within 30–45 minutes (Fig. 1). Ninety minutes after injection — when the estimated cytochrome *c* intracellular concentration⁷ was 20 micromolar — apoptotic morphology was shown by 46.7±4.7% of NRK, 52.6±7.2% of Y-1, 43.5±15.5% of Swiss 3T3 fibroblast and 28.3±4.3% of IPC-81 cells. We verified apoptosis by both co-staining with FITC-conjugated Annexin V and propidium iodide, and electron microscopy (not shown).

This apoptotic effect was found to be due to active cytochrome *c*, as injection of vehicle or of inactive, biotinylated cytochrome *c*⁸ proved ineffective (Fig. 1).

Previously, cytochrome *c* has been shown to trigger caspase activation in cell-free extracts⁴. This conjecture was confirmed when we used cells that had been preincubated (for 30 minutes) with the caspase inhibitor Z-VAD-fmk; at 90 minutes after injection, less than 9.0% were apoptotic (Figs 1, 2c, d).

Cells that overexpressed Bcl-2 were also injected with cytochrome *c*. We found they were partially resistant to induction of killing: at 90 minutes, the level of apoptosis was reduced to approximately 30% of that seen in wild-type cells.

To ensure that we were correctly injecting cells preincubated with Z-VAD-fmk or overexpressing Bcl-2, we co-injected the cells with TRITC and analysed the cells by fluorescence microscopy. The results showed that in both cases cells were properly injected. Thus, the protective effect depended on Z-VAD-fmk treatment

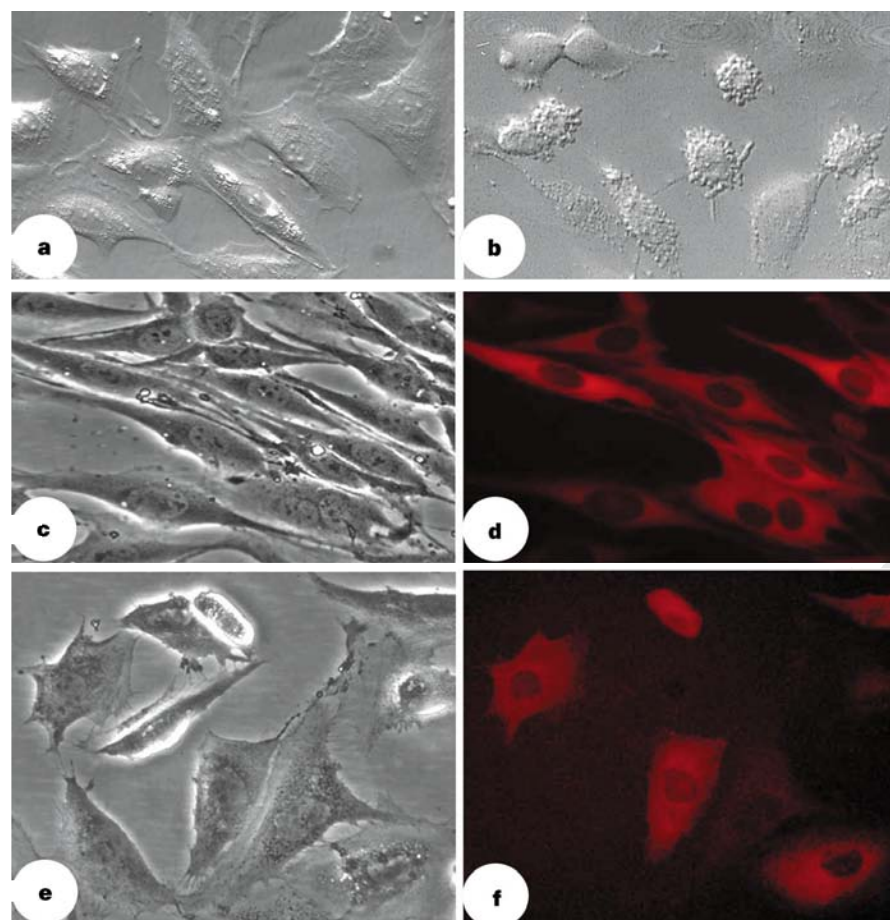


Figure 2 Morphology of microinjected NRK cells. **a**, Control cells. **b**, Wild-type cells injected with 20 μM cytochrome *c*. **c**, Wild type cells preincubated with Z-VAD-fmk for 30 min and co-injected with 20 μM cytochrome *c* and 0.1 $\mu\text{g } \mu\text{l}^{-1}$ TRITC. **d**, Fluorescence micrograph of the same field as that in (c). **e**, Cells overexpressing Bcl-2 co-injected with 20 μM cytochrome *c* and 0.1 $\mu\text{g } \mu\text{l}^{-1}$ TRITC. **f**, Fluorescence micrograph of

and Bcl-2 overexpression, respectively (Fig. 2d, f).

Our results indicate that injected cytochrome *c* can induce apoptosis in various cell types, and that this effect is caspase-dependent. It has been suggested that the role of Bcl-2 protection is restricted to its mitochondrial location, where it prevents leakage of cytochrome *c*^{5,6}. Microinjection of cytochrome *c* bypasses the need for release of this protein from mitochondria. However, cells overexpressing Bcl-2 were also protected from the apoptosis induced by injecting cytochrome *c*. This could be explained in several ways.

First, Bcl-2 is not restricted exclusively to the mitochondrial membrane, hence some of its anti-apoptotic property might be based outside this location¹. Second, Bcl-2 may be a member of the 'apoptosome' complex and it could prevent cell death by direct binding to Apaf-1/Apaf-2/Apaf-3, in a similar way to CED-9's activity on CED-3/CED-4 proteins⁹. Third, it has been reported that cytochrome *c* binds to Bcl-X_L (Ref. 10). Bcl-2 may exert a similar quenching effect outside mitochondria. Fourth, Bcl-2 targets several proteins, including

Raf-1, to mitochondria¹¹. Therefore when overexpressed, Bcl-2 could possibly act as a transporter for cytochrome *c* back into mitochondria¹².

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Tumour regression after endostatin therapy

It was with great interest that we read the report¹ on the lack of acquired drug resistance to repeated doses of endostatin in experimental cancers in mice. Of particular interest is the unprecedented finding that each tumour type became indefinitely dormant after a varying number of treatment cycles. We believe one explanation for this phenomenon might be found in the clinical observation that, after complete regression of large bulky tumours, rebiopsy of the primary tumour site will frequently show large amounts of fibrosis or scarring.

Pharmacological doses of endostatin, a C-terminal fragment of collagen XVIII with relative molecular mass 20,000, at very low concentrations may be deposited in the extracellular matrix with each cycle of therapy, analogous to amyloid deposits in patients with light-chain disease. The marked shrinkage of the mouse tumours from approximately 250–450 mm³ to 5–50 mm³ could effectively increase the local extracellular matrix concentration of endostatin 5–50 fold. Each successive cycle of regrowth followed by treatment and regression could progressively increase the concentration of endostatin in the local extracellular matrix until the concentration is sufficient to inhibit further angiogenesis.

This phenomenon of preferential concentration could explain the observation of a dormant primary tumour while the same tumour type inoculated at a distant site would be uninhibited. The extracellular matrix concentration of endostatin at these distant sites could be several log factors below the concentration at the primary tumour site and insufficient to inhibit tumour-driven angiogenesis.

If this hypothesis is correct, it could mean Boehm *et al.* have serendipitously discovered the ideal way to administer endostatin therapy, delivering a high inhibitory concentration of endostatin at the primary tumour site while keeping the systemic extracellular matrix concentration below the level necessary to inhibit naturally occurring angiogenesis such as wound healing. An assay of the relative concentration of endostatin at the primary tumour site compared to distant tissue concentration should establish if this is the basis for the observed phenomenon.

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Mastering alien etiquette

After Contact: The Human Response to Extraterrestrial Life

by Albert A. Harrison

Plenum: 1997. Pp. 363. \$28.95, £17.55

Phil Morrison

We will know little about extraterrestrials even after prolonged contact. But Albert A. Harrison, professor of psychology at the University of California, Davis, offers us reason enough to justify forecasting what they might be like.

The most striking impression made by this beautifully argued and warm-hearted book is one of subtle unity among humans, extended by probable inference to the 'others'. We are not at all the same, but disparate minds can enrich each other.

The book closes with a terse surprise. The two cultures once perceived by C. P. Snow are now in some degree testable. The Meyer-Briggs type indicator offers an objective measure of Jungian psychological types, and classifies 55 per cent of psychiatrists and social scientists as 'feelers', whereas more than 60 per cent of engineers and scientists are scored as 'thinkers'. Feelers "make decisions on broad personal and social values"; the rest "prefer impersonal, logical bases for decision".

True to type, this physicist reader is a little sceptical. It is far from easy to learn how people make decisions. Perhaps what counts is some weighted sum of life experiences. Harrison's book shows that he shares important traits with feelers, as he focuses easily on emotional responses and will never carelessly hurt another's feelings.

Yet he is attuned to a broader material consistency beyond the richness of social experience. Once he was washed in the sudden light of a fireball as bright as the Moon, another time puzzled by a restored fighter plane from the Second World War that he had mistaken for a model plane flying nearby, and he has long been charmed by the narrow intensities of amateur radio. He cites and follows the rationally structured theory of living systems put forward by James Grier Miller which rests explicitly on continuity among the sciences. His fascinating volume links him to what I conjecture to be the majority among *Nature* readers.

A dozen chapters, each of about 30 pages and almost without visuals, weigh first the physical bases of the search for interstellar signals, then speculations on the likely nature of extraterrestrial society, and finally the human responses we may expect. Harrison offers a well-documented and expressive summary of this magpie literature, avoiding both bland citation and simple dismissal. A too-small sample follows.

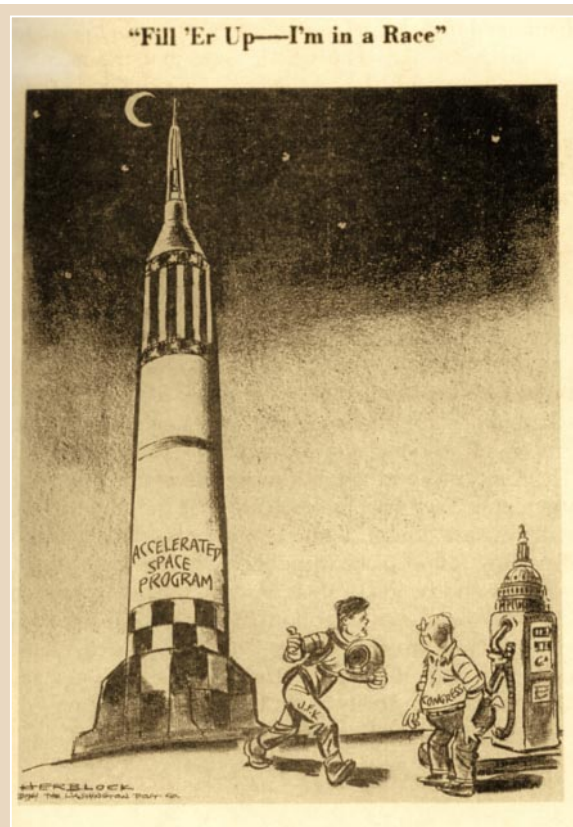
Destination space

This parody of the space race appeared in the *Washington Post* in 1961, and is reproduced in ...*The Heavens and the Earth*, Walter A. McDougall's magisterial political history of the US, European and Soviet space programmes, now reissued in paperback (Johns Hopkins University Press, \$19.95, £15.95). Some ten years on, the author reflects that he should have been gloomier, and that from today's vantage point the space age may well be defined as an era of hubris. To restart it, he says, we must "discover some new principle that makes spaceflight genuinely cheap, safe and routine... however it is achieved, a replacement for the old-fashioned rocket is needed to restore the political will and public support for a second era of 'swashbuckling' in space."

In October 1996 the World-Wide Web contained 20,000 references to unidentified flying objects (UFOs). One form of evidence of extraterrestrial life would be new and verifiable knowledge, so far missing from that haystack of chatter and promises. In Harrison's opinion, the whole UFO effort — perhaps millions of hours — has not added much support to tales from the 1940s. Repeated radio contact is verifiable, and can be shared widely if it comes, to puncture the bubble of false alarms.

Will war follow contact? Some have argued that only one predator society among very many other, benign societies would guarantee disaster. But would one evil empire induce no opposition? A few societies devoted to peace might well prevail. If information is the main basis for exchange across the star-set kiloparsecs, then "hurl threats and I may turn my radio off. Present me with a vision that I like, and I might stay tuned."

Contact will have short-term effects that depend greatly on detailed circumstances. Of the public events that led many to accept the existence of extraterrestrials, only Orson Welles's vivid radio play on Hallowe'en in 1938, as the Second World War threatened, led to a panicky reaction. It aired news of combat against armed Martians in New Jersey. Remakes saw that reaction repeated on several occasions in other countries, most recently in 1988.



But reports in 1835 of carefree lunar 'bat men' acted mainly to sell copies of the hoaxing *New York Sun*. A network of canals on Mars became famous in the 1890s, but gonoliers there were none. Quasars and pulsars — even if controlled by little green men — were mainly science news. Not even the current epidemic of private abductions by UFOs has stirred urgent outcry in the United States.

The most likely form of contact ahead, distant signals from beings unseen and very far away, suggests a brief media circus. The find may come at last to complete our post-Copernican mindset, but only after lengthy decipherment and study.

Harrison ends with some moderate conclusions: if others exist, we will eventually uncover indisputable evidence. They will be recognizable social organisms, and to some extent we will understand them. Our first verifiable contact is more likely to be well handled than it is to incite panic. Their new ideas will not magically cure our ills, but are more likely to help us than to hurt.

So far, I have given only a physicist's paraphrase. But here are his own closing lines: "When we come right down to it, we should place our bets on the side of the optimists.... Let's stay tuned and find out!" □

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The captious crusader

Lancelot Hogben, Scientific Humanist: An Unauthorized Autobiography

edited by Adrian and Anne Hogben

Merlin: 1998. Pp. 254. £14.95

Walter Gratzer

When Julian Huxley published his *Essays of a Biologist*, which duly established itself as a classic blue Penguin shilling paperback, his professed friend and collaborator Lancelot Hogben reviewed it in the following amiable terms: the essays were remarkable chiefly for their "timid superficiality and insipid observation", a consequence of Huxley's "mental immaturity". Francis Bacon said a mouthful when he asserted that friendship is rare in this world, especially among equals; but more than this, Hogben seems to have had a special disposition to tread on any corn in sight. He was a polymath of a type now extinct, possessed of a voracious intelligence which was seldom tempered by any show of modesty; but he also craved recognition, was quick to take offence and nursed a succession of resentments and grievances. He believed that his scientific work was undervalued and his attempts to improve the world thwarted by lesser men.

Yet he threw his eloquence and prestige as a public figure into opposing the pernicious eugenic doctrines that were taking hold in the years after the First World War, and he perceived and denounced the meretricious nature of attempts to link race with intelligence. He was a fine writer and popularizer of science, and his hugely successful books *Science for the Citizen* and *Mathematics for the Million* illuminated the subjects for a mass readership as never before. With Huxley and J. B. S. Haldane he founded the Company of Biologists and the Society for Experimental Biology, complete with its journal, which still thrives today. And in one of his first published papers he demonstrated, against the prevailing view, that chromosomes paired side-by-side in meiosis and not end-to-end.

So in the light of history the old curmudgeon stands well in credit. The biologists of today might be even more impressed to learn that it was Hogben who, during his time in South Africa, introduced *Xenopus laevis*, the indigenous clawed toad, as an experimental animal.

Hogben's elder son (who also became a professor of biology) and daughter-in-law have edited and annotated (perhaps even sanitized?) a mass of unpublished autobiographical material and have given us a prime historical document of science in a socially and politically tempestuous era. More,

Hogben, for all his periodic outbursts of spleen and narcissism, could write with rare vivacity and even charm, most of all about his early life.

Brought up in a comfortless ambience of religious bigotry, threatened with everlasting hellfire should he deviate from the path of physical and intellectual self-denial, the young Hogben found consolation (which endured to the end of his turbulent life) in the study of animals and plants. His rejection of his parents' religion seems to have crystallized from his contempt for the theological dialectic of William Paley and Bishop Butler (who formulated the argument for religious belief, more commonly known now as Pascal's wager, that a prudent man will not take a chance on whether Hell really exists). Hogben relates how on a later occasion he found himself declaiming aloud in the street Swinburne's lines:

*By the name that in hellfire was written, and
burned at the point of the sword,
Thou art smitten, thou God, thou art smitten;
thy death is upon thee, O Lord...*

Hogben won a scholarship to Cambridge where, influenced partly by his first patron, the noted geneticist Leonard Doncaster, he joined the Quakers, "the least pernicious variety of a Christian religion to profess if one feels the need for it". More especially he fell under the sway of the Fabian socialists, notably G. D. H. Cole, for whom (along with the theoretician of the British Communist Party, Raja Palme Dutt) he conceived a great admiration. This did not extend to Cole's followers — a "dreary circuit of smugly self-seeking careerists" — of whom Hugh Gaitskell (who would, had he lived, have been prime minister) emerges as a particular target for Hogben's abundant bile.

Hogben's new-found convictions were to be cruelly tested with the coming of the First World War. He served for six months in a hospital unit in France, but when conscription was introduced he returned to Cambridge to show solidarity with other conscientious objectors and brave the jingoistic passions that by then had pervaded British society. Like Bertrand Russell, he served time in Wormwood Scrubs prison and bravely exposed himself to public and professional obloquy.

The war over, he returned to zoology as a lecturer at Birkbeck College, entered the London *demi-monde* and rose socially. Through G. P. Wells, the zoologist son of H. G., he met the latter's circle, including the Huxleys, and was invited to Lady Ottoline Morrell's salon at Garsington.

By this time he was interested in the new field of endocrinology, and was offered an appointment in Edinburgh in a decaying department headed by the academically moribund E. W. MacBride. MacBride was a vitalist and a Lamarckian, who also urged compulsory sterilization for all males with an annual income of less than £400. Hogben, on



Raffish rogue: Hogben leaves court after being acquitted of a drink-driving charge.

a lecturer's salary of £350, was by then already married and the father of two children.

Not surprisingly he soon moved on to his first professorial chair, at McGill University in Canada. This went well enough until it became known that he had joined the Society for Cultural Relations with the Soviet Union, which, in the eyes of the principal, branded him as a Bolshevik and therefore perforce an adherent of free love. Such a one, the principal informed the dean of the faculty, "could not safely work alone in a laboratory with a lady research assistant". Answering an invitation to a chair at the University of Cape Town, the Hogben *ménage* moved on.

Cape Town offered a cornucopia of rare species to fire the zoologist. Hogben describes how he established a research programme in his department and how he became politically involved through his association with the largely Jewish intelligentsia. His vocal opposition to the increasingly stringent race laws soon ensured his departure for the next and most interesting appointment of his career: already an expert in statistics, he became the first and last occupant of the chair of social biology at the London School of Economics.

It was the wish of the principal, Sir William (not yet Lord) Beveridge, to unite social studies and biology, and Hogben's claims were pressed by the voluble left-wing economist Harold Laski, who was eager to

avert the appointment of one of the many hard-line eugenicists then in the market. Hogben took the job on the condition that he would also be given facilities for laboratory work, and it was thus at LSE, no less, that the first *Xenopus* colony was set up.

Laski, at first a stout ally, later fell into the pit of Hogben's disapproval, as indeed did most of the faculty. Hogben clearly did nothing to assuage hostility either from the left or from the right. One opponent, C. P. Blacker, a respected psychologist with mild eugenic views (not so far from the position of Hogben, who supported the voluntary sterilization of "mental defectives"), has left a description of Hogben at his inaugural lecture, sporting a pink tie, with three curls artfully arranged over his forehead "rather like what you see behind a counter at Selfridges". Nevertheless, Hogben — by many accounts besides his own a rousing lecturer — lauded the sociologists for resisting "the prevalent fashion of biologists to insist exclusively on the genetic factors in social change" and he appealed for collaboration.

Part of Hogben's efforts at this stage went into a new preoccupation with philology. He was a formidable linguist and believed in learning languages by related groups. With one of his South African acquaintances, F. W. Bodmer, he wrote another enduring bestseller, *The Loom of Language*. He also devised a universal scientific language, called Interglossa, which he expounded in a Pelican paperback. His scorn for the established order notwithstanding, he was eager to get into the Royal Society and, believing that popularization was looked on with distaste, he delayed publication of *Mathematics for the Million* until he had been safely elected. Indeed, it became known that he had tried to persuade Hyman Levy, the Communist mathematician and a (transient) friend, to allow the book to appear under his name in place of Hogben's.

But Hogben's credit at the LSE was running out; amidst the customary acrimony he again moved on, this time to a chair at Aberdeen. His sojourn there was interrupted by the Second World War. This time he played an active part, as director of medical statistics for the army. There followed his last academic appointment, that of professor of medical statistics and human genetics at Birmingham, whence he retired to a cottage in Wales. He had divorced his first wife and collaborator of 40 years, Enid Charles, and married a Welsh schoolteacher. But then he accepted the vice-chancellorship of the newly founded University of Guyana. His tenure again came to an abrupt and fiery end, and he returned to his Welsh village.

The death of his second wife left him a lonely and eccentric recluse, given to holding forth in the bar of the local pub to all who would listen, and cherished by the local villagers and hill-farmers. His memoirs end

with reflections on science as it had become by the time of his death in 1975. On the whole he did not much care for what he saw: it was turning, he thought, into a profession dominated by research teams rather than individuals, industry with its demands for secrecy was encroaching, and teaching was becoming more authoritarian. The guiding principle of his youth, that nothing must be taken on faith, was being eroded, and, worst of all, statistics were being deployed by people who had not the faintest understanding of their mathematical basis. Can one imagine anything worse? □

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Going backwards

Time's Arrows and Quantum Measurement

by Lawrence S. Schulman
Cambridge University Press: 1997. Pp. 346.
£60, \$74.95 (hbk); £21.95, \$29.95 (pbk)

Huw Price

Time's arrow and quantum measurement are two of the deepest puzzles of modern physics. In this fascinating book, Lawrence S. Schulman argues that a better understanding of the former problem points to a novel solution of the latter. He first discusses the relationship between the various ways in which time asymmetry appears in physics. His main aim is to defend a proposal of Thomas Gold in the 1950s, that the asymmetry of thermodynamics is due to the expansion of the Universe. Roughly, Gold's idea is that entropy increases because expansion continually raises the bar, creating new possibilities for matter to occupy. So the thermodynamic 'arrow' is a consequence of the cosmological 'arrow'; or "cosmo $\Rightarrow$ thermo", as Schulman puts it.

As Gold saw, it follows from this proposal that entropy would decrease if the Universe recontracted. If expansion implies increase, then contraction implies decrease, for the difference between the two is semantic rather than physical, being no more than a change in sign on the temporal axis. So most cosmologists dismiss Gold's hypothesis, arguing that there is no reason to expect ordinary asymmetric processes to reverse if the Universe recontracts. There is no reason to expect radiation magically to reconverge on stars, or abandoned cups of tea to get spontaneously warmer. For the usual statistical reasons, they say, it is much more likely that entropy would continue to increase.

This use of statistical reasoning is suspect, however. The statistics involved are simply a matter of counting possibilities, and do not distinguish between past and future. On the face of it, then, it is no less unlikely that these

things would happen as we look towards the past than it is that they would happen as we look towards the future. Yet they do happen towards the past! As we look backwards in time we do see radiation 'converging' on stars, and cups of tea getting hotter. If the statistical argument fails so abysmally in this case, why should we trust it to predict the future?

What does this imply for Gold's hypothesis? Good news, in the sense that the standard objection to Gold's time-symmetric Universe (with low entropy at both ends) turns out to be unreliable. But bad news, too, in the sense that Gold's opponents have a valid objection to the claim that expansion is the key to the thermodynamic asymmetry. In showing that the Universe could quite well recontract without a decrease in entropy, they show that it could expand without an increase in entropy. (Again, this is the very same thing, in different words.) The key to the thermodynamic arrow must lie elsewhere, perhaps in some sort of 'boundary condition' requiring that the Universe be in a special state at one or both ends.

Schulman does not fully appreciate this bad news, I think, but his discussion of the good news — the possibility of symmetric cosmological models, with two-time boundary conditions — is full of insight.

But the most interesting and novel part of the book is his proposed solution to the quantum measurement problem. When extrapolated to macroscopic systems, the dynamics of quantum mechanics allow the state of a system to evolve into 'grotesque' superpositions of macroscopically distinct possibilities, such as a superposition of a dead and alive cat. Wave-function 'collapse' eliminates such embarrassments, but at terrible cost. After 70 years, there is still no adequate understanding of what this collapse is, or when it happens.

Schulman's suggestion is that in all cases in which such grotesque states threaten there are some 'special' initial states, compatible with whatever constraints the experimental circumstances impose, which do not evolve into grotesque states; and that the actual initial state is always one of these special states. In terms of dynamics this is just orthodox quantum theory, without collapse. States always evolve in accordance with Schrödinger's equation. But, exploiting our limited control over initial set-ups, nature always arranges things so that the grotesque possibilities do not happen.

Schulman presents this proposal by analogy with Gold's symmetric cosmology, in which future boundary conditions imply microscopic 'hidden order' in present-day systems, the kind of vast hidden correlations required for radiation eventually to reconverge on stars, for example. In the quantum case, too, Schulman thinks, we need a future boundary condition to explain why quantum systems choose their states from a tiny

subset of the available alternatives.

But there may be an easier solution. Non-special states lead to such absurdities as alive-and-dead cats. Why not take this as a demonstration that these states are not really possible after all? This would leave the (now misnamed) special states as the only options, and would leave nothing for future conditions to explain.

The link between time's arrow and quantum measurement might thus be less close than Schulman suggests. Nevertheless, his book is essential reading for anyone with a serious interest in either topic. □

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Faulty weather

Tectonic Uplift and Climate Change

edited by W. F. Ruddiman

Plenum: 1997. Pp. 535. \$115, £83.64

Jean-Pierre Burg

There is a surrealist French song that laments the impossible love between a fish and a bird. It is a poetic stance reflecting that until recently the 'material' earth and the 'immaterial' atmosphere were thought to be worlds even more estranged than scientific orthodoxy suggested.

But Charles Lyell believed that the creation of recent mountains influenced the climate. Since then, modelling using non-classical statistics has opened up previously unimagined horizons. If a butterfly flapping its wings in the Amazon forest can trigger frightful storms over Europe, is it so hard to accept that a rising mountain has its own climatic might?

W. F. Ruddiman has put together a well-integrated answer for scholars and graduate students. It is built on geological information, palaeoclimatic records, ideas developed from a detailed knowledge of elevated regions and surrounding areas, and numerical modelling. The authors provide the pertinent yet equivocal evidence behind the hypothesis linking uplift with climate, and subsequent controversies.

Large-scale topographic changes result from tectonic convergence or extension and subsequent isostatic compensation. In the first chapters, three examples (South-East Asia, the central Andes and east Africa) provide information about the long-term links between global climate and the uplift of large areas. Elevated plateaux and collisional mountains are physical obstacles to wind and oceanic circulation. Over the past year El Niño has reversed its course, providing a timely reminder of the short-term effects of circulation changes.

The importance of numerical experiments for understanding geologically relevant effects is developed in the next chapters,



Gully erosion in the badlands of South Dakota. From the second edition of *Understanding Earth* by Frank Press and Raymond Siever (W. H. Freeman, \$62.95, £25.95, pbk; see *Nature* 370, 261; 1994).

which are mostly original in content. Theoretical knowledge gained from atmospheric sciences, applied to problems over large scales of time and space, helps to isolate the effects of mountains from other climatic components.

Many readers will learn that the unusually high present-day mountains can cause drying of mid-latitude continental regions. Many more will be intrigued by the relationship between orographic changes, resulting from increased physiographical gradients, and changes in the carbon cycle. Indeed, more than half of the volume deals with the balance between the rates of physical weathering — which includes orographic changes and thus the drainage capacity of high mountainous terrains — and chemical weathering — which includes the effects of dissolved loads in rivers and their capacity to extract from rocks the chemical elements that control oceanic and atmospheric compositions. These factors have an indirect but extensive effect on the global climate, mostly through the greenhouse effect.

Because of its size, the Tibet–Himalaya region is an important case study. Its effect on the climate of the Northern Hemisphere seems certain. But how does a rising region increase erosion, alter global circulation and affect seasonal distribution and contrasts? Why might these modifications increase global mean rates of chemical weathering, which could reduce carbon dioxide levels and thus further affect the climate, and so on?

Scientists will get ideas from reading this book. They will also find interdisciplinary research that has been successful in stimulating new thought and targeting important issues, and which emphasizes the role of such interdisciplinary research in science today.

Einstein argued that time and space are like a theatre performance: the action starts from nothing, then the relationship between the actors defines the stage. Similarly, tectonics and global changes are intricately

associated to enliven our planet.

A few years ago, *Tectonic Uplift and Climate Change* would have been a science-fiction title, if not a heresy. Today, and thanks to syntheses such as this, we suspect that there may be more than a casual relationship between the evolving topography and the climate. □

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ADVERTISEMENTS

Integrin-mediated short-term memory in *Drosophila*

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***Volado* is a new memory mutant of *Drosophila*. The locus encodes two isoforms of a new α -integrin, a molecule that dynamically mediates cell adhesion and signal transduction. The *Volado* gene is expressed preferentially in mushroom body cells, which are neurons known to mediate olfactory learning in insects. *Volado* proteins are concentrated in the mushroom body neuropil, brain areas that contain mushroom body processes in synaptic contact with other neurons. *Volado* mutants display impaired olfactory memories within 3 min of training, indicating that the integrin is required for short-term memory processes. Conditional expression of a *Volado* transgene during adulthood rescues the memory impairment. This rescue of memory is reversible, fading over time along with expression of the transgene. Thus the *Volado* integrin is essential for the physiological processes underlying memory. We propose a model in which integrins act as dynamic regulators of synapse structure or the signalling events underlying short-term memory formation.**

Behavioural and cellular studies have revealed two broad phases of memory: short-term and long-term memory. Short-term memory, which lasts from minutes to hours, is thought to occur through changes in synaptic efficacy produced by rapid and transient biochemical alterations in the relevant neurons^{1–5}. In contrast, long-term memory, which lasts from days to years, is thought to occur through changes in synaptic efficacy produced by the restructuring of synapses as a result of altered gene expression^{6–10}. The formation of long-term, but not short-term, memory has therefore been thought to rely upon morphological restructuring of synapses using mechanisms similar to those involved in brain development.

In *Drosophila*, the formation of olfactory memories is scripted in cyclic AMP signalling in neurons of the mushroom bodies^{4,11,12}. Studies linking cAMP signalling, mushroom bodies, and olfactory learning demonstrated the preferential expression in mushroom bodies of three genes required for normal learning: *dunce* (*dnc*), *rutabaga* (*rut*) and *DCO* (the genes encoding cAMP phosphodiesterase, adenylyl cyclase and the catalytic subunit of protein kinase A (PKA), respectively)¹¹. Moreover, the characterization of two other learning genes (*amnesiac* and *DCREB2*) of *Drosophila* is consistent with a dominant role for cAMP in modulating the physiology of neurons that mediate behavioural plasticity. The *amnesiac* gene encodes a peptide similar to pituitary adenylyl cyclase-activating peptide¹³ and *DCREB2* encodes a transcription factor that may mediate cAMP-dependent gene expression¹⁴. These studies suggest that mushroom bodies function as the integration and memory centre for olfactory learning by using the cAMP signalling system^{4,11}.

Despite the evidence pointing to the cAMP signalling system, many different types of molecules must be engaged during learning to bring about the overall physiological changes in the relevant neurons. Indeed, the involvement of an assortment of protein kinases, transcription factors, enzymes involved in neurotransmitter biosynthesis, neuropeptides and other factors have been suggested^{5,15–19}. Here we report the isolation of a new *Drosophila* memory gene, *Volado* (*Vol*). (*Volado* is a Chilean colloquialism with no English counterpart, but is loosely translated as 'forgetful' or 'absent-minded'. In Chile, it is often used in reference to professors and scientists.) *Vol* encodes a new α -integrin, a type of cell-surface receptor known to dynamically mediate cell adhesion and signal transduction²⁰. Lesions in *Vol* have a dominant effect upon short-term memory following olfactory conditioning. Remarkably, conditional expression of *Vol* just before training rescues the memory

deficit of *Vol* mutants. This rescue is reversible, supporting a dynamic role for integrins in neuronal and behavioural plasticity. These data indicate that integrin-mediated signalling or synaptic restructuring underlie the formation, stability or retrieval of short-term memory.

The *Vol* locus encodes new α -integrins

The importance of mushroom bodies in olfactory learning had previously prompted us to construct and screen ~6,000 enhancer detector lines for preferential expression of the *lacZ* reporter in these brain structures²¹. About 100 lines with preferential mushroom body expression were isolated, including insertions at the *dnc*, *rut*, *DCO* and *leonardo* (*leo*) genes^{11,22}. Line 1,116 (*Vol*¹) from this screen also expressed *lacZ* in mushroom bodies; the enhancer detector element in this line was mapped to cytological position 51E.

We isolated the region flanking the enhancer detector element along with wild-type genomic and cDNA clones for the locus. The locus is organized into two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), which encode RNAs of 4.6 kilobases (kb) and 4.4 kb, respectively (Fig. 1b). The *Vol*-l RNA is expressed selectively in heads, whereas *Vol*-s is expressed in both head and body tissues (not shown). Mapping experiments showed that the *Vol*¹ enhancer detector element resides within the first intron of *Vol*-l and within the 5' flanking region of *Vol*-s (Fig. 1a). Imprecise excision of the element led to the isolation of *Vol*², an allele with a deletion of 816 nucleotides of genomic sequence that removes the first exon of *Vol*-s (Fig. 1a). Reverse-transcription-polymerase chain reaction (RT-PCR) analyses of head RNA revealed that expression of the *Vol*-l transcript was greatly reduced in *Vol*¹, but the *Vol*-s transcript was unaffected (Fig. 1c). Conversely, the *Vol*² lesion eliminated the *Vol*-s transcript without discernible changes in *Vol*-l (Fig. 1c). Neither allele affected the expression of PKA, the internal control in these experiments (Fig. 1c). The effects of the alleles on expression of the two transcripts, as confirmed by RNA blotting experiments (not shown), are consistent with the nature of the physical lesions at the gene (Fig. 1a). Thus the *Vol*¹ and *Vol*² alleles disrupt the expression of *Vol*-l and *Vol*-s, respectively.

The cDNAs for *Vol*-l and *Vol*-s predict new α -integrins of 1,115 amino acids, differing only in the first 63 amino acids (Figs 1a, 2). *Vol* proteins contain many characteristics of other α -integrins²⁰. *Vol* proteins are 23–28% identical in amino-acid sequence with known α -integrins and contain a single transmembrane domain near the carboxy terminus. The proteins begin with 24 residues of a hydrophobic, putative signal peptide, have 11 potential glycosylation sites [NXT(S)] in the extracellular region, and have three repeats in the

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extracellular region that match the consensus DX(D/N)X(D/N)GXSD, a domain found in proteins that bind divalent cations²³. Moreover, the Vol sequence has a cleavage recognition site (RKRR) in the extracellular domain²⁴, a site required for signal transduction by some α -integrins. After cleavage at these sites, the amino-terminal and carboxy-terminal integrin fragments are held together by disulphide bonds^{20,25}. Furthermore, the cytoplasmic domain of Vol contains the consensus sequence KXFF[K/R]R, which binds calreticulin²⁶ and regulates integrin affinity for ligand.

Expression of Vol in mushroom bodies

The Vol¹ mutant preferentially expressed the lacZ reporter in the nuclei of mushroom body neurons (Fig. 3a). To determine whether the enhancer detector reflected authentic Vol expression, we performed immunohistochemical analyses with an antiserum made against the C terminus of the protein. The Vol antigen was found to be concentrated in the mushroom body perikarya and calyces (Fig. 3b), peduncles (Fig. 3c) and α , β and γ lobes (Fig. 3d). The calyces, peduncles and lobes contain the mushroom body dendrites, axons and axon terminals, respectively¹¹. The distribution of the antigen was not noticeably altered in the Vol¹ or Vol² mutants (data not shown), suggesting that both the Vol-l and Vol-s isoforms are globally coexpressed in the mushroom bodies. Enriched expression was also observed in the ellipsoid body (not shown), a region of the central complex thought to be involved in the coordination of motor behaviours²⁷. The distribution of Vol in the mushroom body calyces and lobes (regions in which mushroom body neurons form

synapses with other neurons) suggests that the Vol integrins may regulate synapse function.

Vol mutations produce a memory deficit

The expression pattern of Vol, coupled with preliminary behavioural experiments, suggest that the gene is important for olfactory memory. To test this hypothesis, we assayed Vol mutants for aversive olfactory classical conditioning⁴. Populations of animals were administered electric shock (unconditioned stimulus; US) in the presence of one odour, the conditioned stimulus (CS⁺), and were subsequently presented with a second odour (CS⁻) without shock. To evaluate discriminative avoidance behaviour, the trained animals were allowed to distribute between converging CS⁺ and CS⁻ odours carried in air currents within a T-maze.

Animals homozygous for the Vol¹ insertion or the Vol² deletion performed poorly relative to rosy (ry) control flies at all time points after training (Fig. 4a; genotype, $P = 0.0001$; retention interval, $P = 0.0001$; genotype $\times$ retention interval, n.s.; see Supplementary Information for the complete statistical treatment for these and

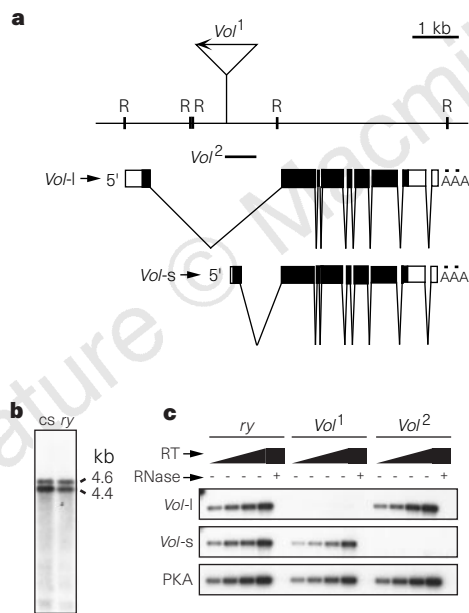


Figure 1 The Vol gene structure, transcripts and mutations. **a**, An EcoRI (R) restriction map of the locus is shown with the position of the Vol¹ enhancer detector element indicated by a triangle. The direction of transcription of the lacZ reporter in the enhancer detector element is indicated by the arrow. Two transcription units, Vol-long (Vol-l) and Vol-short (Vol-s), were deduced by comparing cDNA sequences with genomic sequences. The first exon of each transcription unit is spliced to a common 2nd exon. Filled boxes represent the open reading frame. The 816 base-pair deletion in Vol² is indicated by the line spanning the first exon of Vol-s. **b**, Blots of adult head RNA showing the 4.6- and 4.4-kb transcripts of Vol-l and Vol-s, respectively, in Canton-S (cs) and ry animals. **c**, RT-PCR analyses of total head RNA from rosy (ry), Vol¹ and Vol² adults. Each graded bar represents increasing amounts (from left to right) of a single RT reaction added to the subsequent PCR. Both Vol-l and Vol-s were present in ry; however, the expression of Vol-l was dramatically reduced in Vol¹ and expression of Vol-s was undetectable in Vol². The internal control using PKA primers allowed quantitative comparisons to be made between the various RT-PCR reactions. RNase (+) added before the RT reaction abolished all signals.

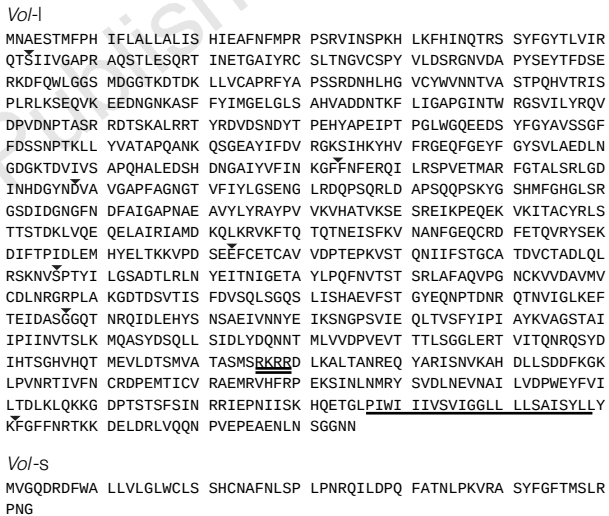


Figure 2 Vol encodes two forms of an α -integrin. The complete predicted amino acid sequence for Vol-l and the first 63 amino acids of Vol-s are shown. Triangles above the sequence indicate where the coding sequence is separated by introns. The putative transmembrane domain and cleavage site are indicated by single and double underlines, respectively.

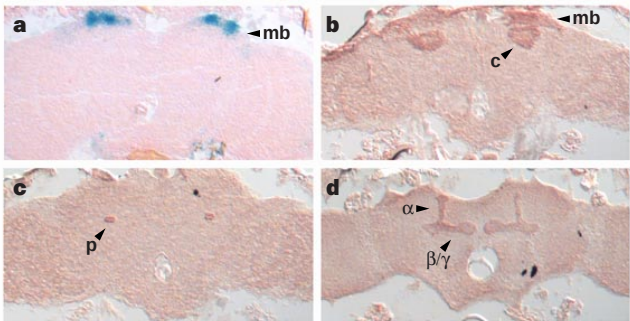


Figure 3 Vol is preferentially expressed in mushroom bodies. **a**, Frontal section of a Vol¹ adult head stained for β -galactosidase activity. Staining (blue) was observed within the mushroom body perikarya (mb). The β -galactosidase encoded by the enhancer detector element carried a nuclear targeting sequence which explains the nuclear localization of the histochemical stain. **b-d**, Frontal sections of Canton-S adults after immunostaining with an affinity-purified antiserum raised against the C terminus of Vol. Expression (dark brown) was observed in: **b**, the cell bodies (mb) and calyces (c); **c**, the peduncle (p); and **d**, the α , β and γ lobes (α , β , γ).

other data presented below). The effects of these mutations on memory were indistinguishable, suggesting that the two integrin isoforms are functionally redundant. We have shown previously that neither the enhancer detector itself nor the expression of *lacZ* in mushroom bodies has any significant effect upon performance (ref. 22 and K.H.W. and R.L.D., unpublished). The performance deficits in *Vol* mutants were present at the earliest testable time point after training (3 min), indicating that the formation, stability or retrieval of short-term memory is dependent on integrin function.

To examine further the effects of the *Vol* alleles on early memory, and to investigate their recessive or dominant nature, we trained and tested the performance of animals heterozygous or homozygous for the two lesions of the gene. The *Vol¹* and *Vol²* animals exhibited memory deficits at both 3 and 15 min after training (Fig. 4b; 3 min, $P = 0.0001$; 15 min, $P = 0.0001$), confirming the results in Fig. 4a. The performance indices of the *Vol¹/+* and *Vol²/+* heterozygous animals were similarly reduced relative to *ry*, but were not significantly different from the corresponding homozygous mutants (Fig. 4b; *Vol¹* versus *ry* at 3 min, $P = 0.0001$; at 15 min, $P = 0.0001$; *Vol²* versus *ry* at 3 min, $P = 0.00015$; at 15 min, $P = 0.0001$; *Vol¹* versus *Vol¹/+* at 3 min, n.s.; at 15 min, n.s.; *Vol²* versus *Vol²/+* at 3 min, n.s. at 15 min, n.s.). Trans-heterozygous animals, *Vol¹/Vol²*, also exhibited a performance index equivalent to *Vol¹/+* or *Vol²/+* (not shown). Thus, as with *dnc*, *rut*, *turnip*, *radish* and *cabbage*, mutations in *Vol* have a dominant effect on memory⁴. The dominant effect is particularly noteworthy for *Vol* alleles, as three of the four transcription units were preserved in animals heterozygous for *Vol-1* or *Vol-s* lesions. These data support the existence of a threshold requirement for *Vol* expression in the processes underlying memory, making them acutely sensitive to decreased expression of this gene.

To eliminate the possibility that the poor performance of *Vol* mutants was due to defects in sensorimotor processes, we tested their ability to sense and avoid electric-shock pulses and the odours

used for conditioning⁴. The avoidance behaviour of *Vol* mutants and control animals to electrified grids and odours used for conditioning at multiple strengths of these stimuli was indistinguishable. For example, the avoidance indices to 0.8 ml octanol were 63 ± 4 , 68 ± 4 and 65 ± 5 for *ry*, *Vol¹* and *Vol²*, respectively. (See Supplementary Information for the complete olfactory and electroshock avoidance data.) We also explored the morphology of the brain, with particular emphasis on mushroom bodies, to determine whether the poor performance was attributable to defects in brain structure. Serial paraffin sections of control and mutant brains failed to reveal any discernible differences in morphology when stained with haematoxylin and eosin, an antibody against the nuclear antigen D-Mef2, which reveals a subset of mushroom body cell nuclei (J. Crittenden and R.L.D., unpublished); an antibody against the *leo* gene product, which delineates the mushroom body calyces, cell bodies, peduncles and lobes²²; or an antibody against FasII, which reveals a subset of the mushroom body lobes (Fig. 5 and data not shown). Therefore, neither sensorimotor nor gross neuroanatomical defects can account for the memory deficit of *Vol* mutants.

Conditional rescue of the *Vol* memory deficit

We reasoned that direct evidence for a role of the integrin in physiological processes underlying memory might be obtained by the conditional expression of a *Vol* transgene. Four transgenic lines were generated that contained the *Vol-s* cDNA under the control of the heat-shock promoter (*hsp70*) in the *Vol²* background (*Vol-s* mutant). Animals were heat-shocked for 15 min at 37°C, rested for 3 h to allow recovery and expression of the transgene, and were trained and tested for 3-min memory. Two of the transgenic lines failed to show any evidence of heat-dependent rescue in pilot experiments, presumably owing to genomic position effects, and were not analysed further. Two other lines, VS-T2 and VS-T3, were analysed extensively for olfactory memory.

Normal olfactory memory of *ry* control animals and the residual memory in *Vol²* mutants was unaffected by heat shock (Fig. 6a; for *ry*, no heat shock versus heat shock, 3 h, n.s., for *Vol²*, no heat shock versus heat shock, 3 h, n.s.). In the absence of heat shock, VS-T3 transgenic animals exhibited mutant levels of performance, but VS-T2 transgenic animals showed partial rescue of memory, possibly

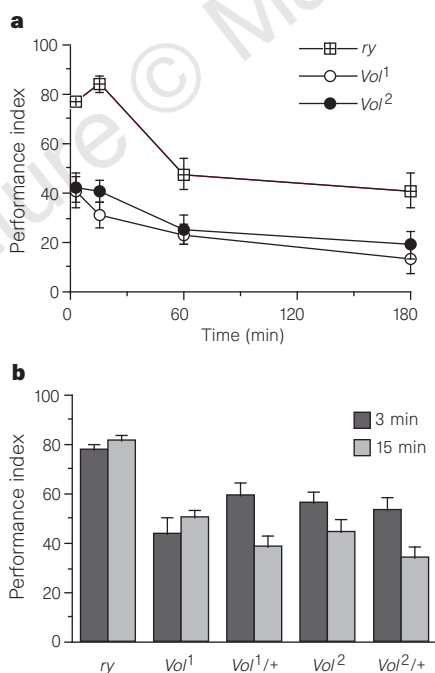


Figure 4 Memory deficits in *Vol* mutants. **a**, Decay curve of conditioned odour avoidance for two *Vol* mutants (*Vol¹* and *Vol²*) and the control strain (*ry*); $N = 8-9$ for all groups. The mean performance index $\pm$ s.e.m. is shown for each genotype at several time points after training. The performance of *Vol¹* and *Vol²* was significantly less than *ry* at all time points. **b**, Performance of homozygous and heterozygous *Vol* mutants at 3 and 15 min after training; $N = 8-11$ for all groups. There were no significant differences between the homozygous mutant strains and the corresponding heterozygous strains at either time point.

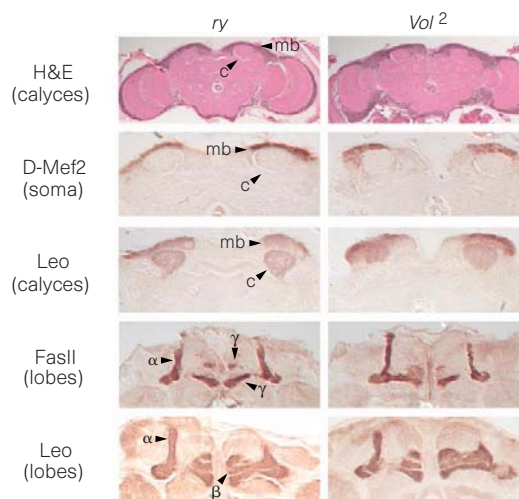


Figure 5 Lack of neuroanatomical defects in *Vol* mutants. Frontal sections from *ry* and *Vol²* adult flies are shown at the level of the mushroom body perikarya (mb) and calyces (c) after staining with haematoxylin and eosin (H&E) or with an antibody against the nuclear antigen D-Mef2, and at the level of the mushroom body lobes (α , β and γ) after staining with anti-FasII or anti-Leonardo antisera. No differences were observed between *ry* and either mutant (*Vol¹* not shown). Slight differences seen here were due to the plane of sectioning. The posterior to anterior arrangement of sections is from top to bottom.

owing to elevated basal expression of the transgene in mushroom bodies (Fig. 6a; no heat shock, VS-T3 versus *ry*, $P = 0.0001$; VS-T3 versus *Vol*², n.s.; VS-T2 versus *ry*, $P = 0.0008$; VS-T2 versus *Vol*², $P = 0.0003$). However, the 3-min memory of VS-T2 and VS-T3 animals when trained 3 h after heat shock was significantly greater than after no heat shock, and was indistinguishable from the *ry* control (Fig. 6a; heat shock versus no heat shock, VS-T2, $P = 0.0045$; VS-T3, $P = 0.005$; with heat shock, *ry* versus VS-T2, n.s.; *ry* versus VS-T3, n.s.). Therefore, conditional expression of *Vol*-s just before behavioural training was sufficient to fully rescue the mutant phenotype. This rescue cannot be attributed to altered sensorimotor abilities because avoidance behaviour to electric shock and odours by the control and transgenic animals was indistinguishable, with or without heat shock (see Supplementary Information). These data argue that the defective α -integrin expression in *Vol* mutants causes the memory deficits, and that the *Vol* integrin participates in the physiological processes underlying memory.

To determine whether the behavioural rescue was paralleled by the induction of the *Vol* transgene, we assayed *Vol* RNA and protein levels before and after heat shock. As assayed by RT-PCR, heat shock

had no effect on the quantity of *Vol*-s RNA in *ry* control animals (Fig. 6b), but produced ~100-fold and ~1,000-fold increases in the level of *Vol*-s RNA in the VS-T2 and VS-T3 transgenic lines, respectively (Fig. 6b). The level of PKA RNA served as an internal control and was unaffected by *Vol* mutation (Fig. 1b), *Vol* transgene expression, or heat shock (Fig. 6b). To measure Vol protein, we performed western blotting experiments using an affinity-purified antiserum raised against the C terminus of Vol that recognized the intact Vol protein (relative molecular mass 125,000; $M_r \sim 125$ K) and the C-terminal cleavage fragment produced by proteolysis ($M_r \sim 21$ K). This antiserum identified a band (sometimes a doublet) of 26 K in *ry* that was not found in *Vol*² mutants or in non-heat-shocked transgenic animals (Fig. 6c and data not shown). This band represents the C-terminal cleavage fragment. The full-length protein was not detected in *ry* extracts, presumably owing to reduction of the disulphide bond that links the heavy and light chains. In contrast to the *ry* control, a large increase in the expression of both the *Vol* full-length protein and light chain was found in VS-T3 extracts obtained 3 h after heat shock (Fig. 6c). Detection of the intact molecule suggests that the protease is limiting after overexpression of *Vol*. Induction of Vol was also observed in VS-T2 (not

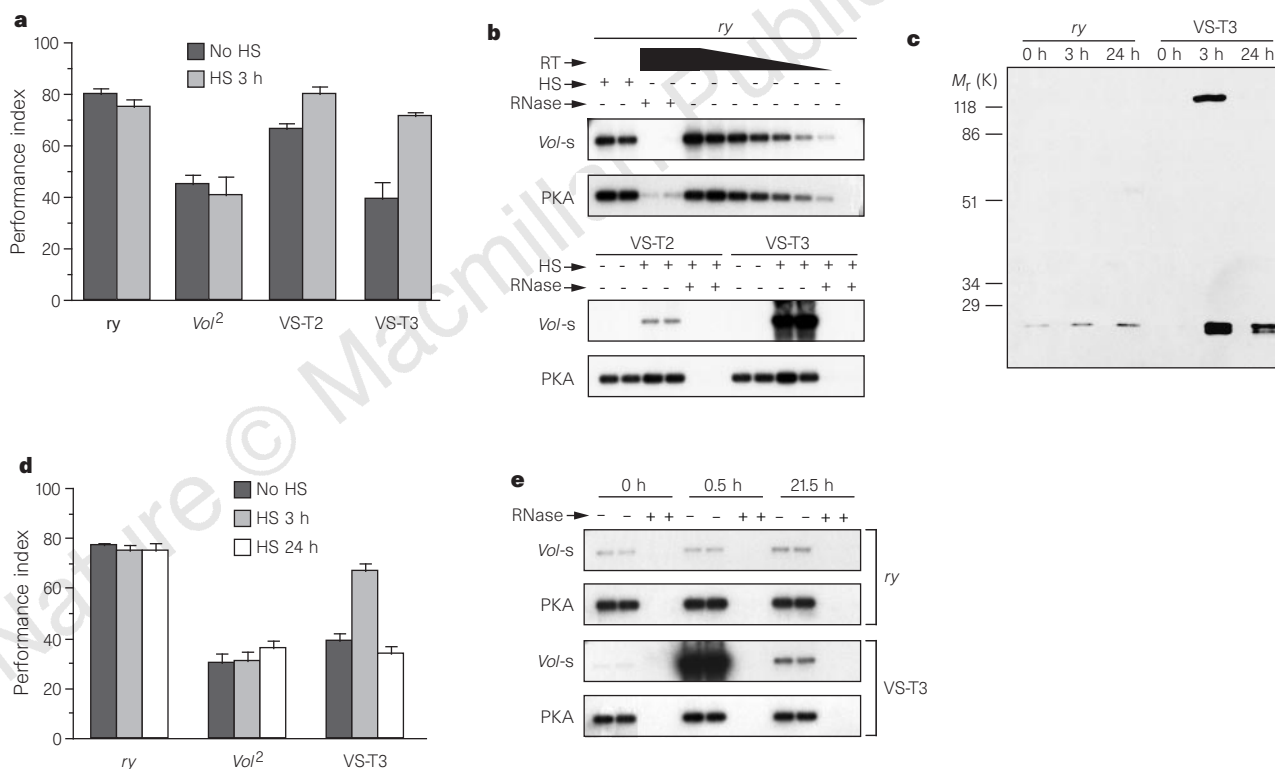


Figure 6 Rescue of the *Vol* memory defect by conditional expression of *Vol*-s. **a**, Memory after 3 min, without heat shock (No HS) or with training 3 h after heat shock (HS 3 h) in *ry*, *Vol*², VS-T2 and VS-T3 flies. Heat shock was for 15 min at 37 °C; $N = 6$ for all groups. Rescue of the mutant phenotype was exhibited by both VS-T2 and VS-T3; VS-T2 exhibited some constitutive rescue. **b**, RT-PCR analyses of *Vol*-s expression. RT-PCR for *ry*, VS-T2 and VS-T3 without (–) or 30 min after (+) HS. Top, *ry* control: HS had no effect on expression of *Vol*-s or PKA in *ry* animals (compare duplicate lanes 1 and 2 with lane 7, all of which are from PCR reactions containing equivalent amounts of input cDNA). Quantification using a BetaGen blot analyser demonstrated that signals for both *Vol*-s and PKA were linear with the mass of input cDNA amplified by PCR. Bottom, *Vol* transgenics: *Vol*-s RNA was nearly undetectable in both VS-T2 and VS-T3 in the absence of HS (–). There was a marked induction of the transgene 30 min after HS (+). Lanes 1, 2 and 7 in the top panels and all lanes in the bottom panels are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment (+) before RT eliminates all signals. Data are representative of 3 independent experiments. **c**, *Vol* protein was induced after HS in VS-T3. Immunoblotting was performed on extracts from whole

flies without (0 h) or 3 and 24 h after HS. Western blots containing 0.5 fly equivalents per lane were incubated with an affinity-purified antiserum generated against the C-terminus of the Vol integrin. This antiserum recognizes both the full-length protein (~135K) as well as the light chain (doublet at ~26K). These data, confirmed by detection with an antiserum generated against the extracellular domain (not shown), are representative of 2 experiments. **d**, Memory at 3 min without HS (No HS) or 3 (HS 3 h) and 24 h (HS 24 h) after HS; $N = 6$ for all groups. VS-T3 showed a behavioural deficit without HS, normal performance with HS 3 h before training, but a deficit again when HS was given 24 h before training. **e**, RT-PCR analyses of *Vol*-s RNA expression without (0 h) or 0.5 and 21.5 h after HS. Top, Expression of *Vol*-s was not changed after HS in *ry* animals. Bottom: *Vol*-s RNA was dramatically elevated in VS-T3 0.5 h after HS, and returned to a low level at 21.5 h after HS. As in **b**, PKA expression was not changed by HS in either strain. All lanes are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment before RT eliminates all signals. Data, from a single experiment performed in duplicate, are representative of 2 independent experiments.

shown). Thus there was a marked elevation of the *Vol* α -integrin in the VS-T2 and VS-T3 transgenics 3 h after heat shock. These RNA and protein analyses demonstrated that *Vol* was conditionally expressed at the time of behavioural assay, confirming that replacement of the *Vol* integrin in adulthood rescued the memory deficit.

Our data point to a physiological role for *Vol*; however, it seemed plausible that the α -integrin might be required for a final step in synapse formation that occurs normally during development, and that the induced expression of the integrin during adulthood simply allows completion of this terminal step. In other words, the integrin might be required for synapse formation but not for synapse stability. If this were the case, the induction of *Vol* expression might cause a long-lasting or permanent rescue of memory. However, if *Vol* participates in a non-developmental, acute aspect of neuronal function, the rescue of memory produced by induction of the *Vol* transgene would be expected to be transient and reversible, persisting only as long as adequate levels of the *Vol* integrin were present.

To distinguish between these possibilities, we explored whether induction of *Vol* produced a permanent or a reversible restoration of memory. As before (Fig. 6a), heat-shock treatment 3 h before training and testing dramatically improved the performance of VS-T3 animals (Fig. 6d; for VS-T3, no heat shock versus heat shock, 3 h, $P = 0.0001$). This rescue was completely reversible. The memory in heat-shocked VS-T3 transgenic animals returned to mutant levels when the animals were trained and tested 24 h after heat shock (Fig. 6d; for VS-T3, no heat shock versus heat shock, 24 h; n.s.; heat shock 3 h versus heat shock 24 h, $P = 0.0001$; for heat shock 24 h, *ry* versus VS-T3, $P = 0.0001$; *Vol*² versus VS-T3, n.s.). *Vol* RNA and protein expression in the transgenic animals, which reflect abundance in all cells, were markedly elevated at early time points after heat shock (0.5 and 3 h, respectively), and decreased to low levels at late time points (21.5 and 24 h, respectively) (Fig. 6c, e). Thus the induction and ensuing decline of *Vol* expression correlated well with the behavioural rescue and subsequent return to a state of memory impairment. The temporal parallels in RNA level, protein expression, and memory argue strongly that *Vol* mediates a physiological process that is critical to memory formation, stability or retrieval.

Taken together, our data support three important points. First, reduced expression of the *Vol* integrin produces an impairment in memory without altering sensorimotor abilities or neuroanatomy. Second, this phenotype is rescued by the expression of the integrin just before training in the adult animal, demonstrating a role in adults for this adhesion molecule. Third, the reversibility of the memory rescue indicates that the *Vol* integrin mediates a dynamic process underlying memory.

Role of integrins in plasticity

The identification, isolation and characterization of *Vol* follows previous studies of four other learning genes with similar expression patterns: *dnc*, *rut*, *DCO* and *leo*^{11,22,28–30}. The discovery of another memory mutant in which the underlying gene is expressed preferentially in mushroom bodies reinforces the conclusion that these cells are required for olfactory learning and memory. Mushroom bodies have been the centre of physiological, anatomical and behaviour studies in large insects^{11,12}, and have since been shown to be important in *Drosophila* behaviour¹¹. One attractive model has mushroom bodies as centres for the reception and integration of many different forms of sensory information, including information about odours and electric shock presented during olfactory classical conditioning. To encode memory, the converging sensory information is thought to alter the physiology of mushroom body cells, using the cAMP signalling system as well as other types of molecules^{4,11,22,31}. Our results demonstrate that integrins should now be included in the family of molecules required for memory formation.

Integrins have diverse biological roles in apoptosis, cell-cycle

regulation, cell migration, blood clotting and leukocyte function^{20,25,32,33}. They function as $\alpha\beta$ heterodimers, mediating adhesive interactions of cells with the extracellular matrix or with counter-receptors displayed by other cells. They also dynamically transduce information across cell membranes bi-directionally^{20,25,32,33}. Ligand binding to integrins induces a variety of signalling events within cells, and agonist activation of classical signal-transduction pathways can alter the affinity of integrins for their ligands within just a few minutes²⁵.

The dynamic adhesion role for integrins suggests how the *Vol* integrin, and integrins in general, may underlie alterations in synaptic plasticity and behaviour. We envisage that release of a modulatory neurotransmitter on a mushroom body neuron might mobilize the intracellular events altering the binding of integrins displayed at another synapse made by that cell. For example, protein kinase C or Ras activation^{20,32,34} is known to activate integrin binding. This could produce a rapid (within minutes) alteration in the structure and efficacy of that synapse. The modulation of integrin affinity for ligands might also underlie the construction or pruning of existing synapses, or the activation of silent synapses during learning or memory encoding. Thus the formation of short-term memory may use synaptic rearrangements like long-term memory, but through an integrin-dependent and protein synthesis-independent mechanism. Alternatively, integrins might modulate neuronal function through ligand binding followed by activation of intracellular signalling events. For example, integrins are known to stimulate several signal-transduction pathways in many types of cells, including Ca^{2+} mobilization, tyrosine kinase activation, and induction of protein kinase C³³. Integrin-dependent stimulation of these pathways in the relevant neurons may be fundamental to learning and memory.

Our results demonstrating a role for integrins in behavioural plasticity fit well with studies showing integrin-dependent modulation of synaptic plasticity. Notably, peptide inhibitors of integrin binding have no effect on the formation of long-term potentiation, but block the maintenance of this form of synaptic plasticity^{35,36}. In addition, the enhancement of neurotransmitter release from motor-neuron terminals owing to muscle stretch is blocked by peptide inhibitors³⁷. These physiological studies³⁸, coupled with our behavioural studies, support a model in which integrins mediate dynamic processes at synapses underlying memory formation or stability.

Note added in proof: A paper reporting on another aspect of the same novel integrin subunit is in press (Stark, K. A., Yee, G. H., Roote, C. E., Williams, E. L., Zusman, S. & Hynes, R. O. A novel α integrin subunit associates with β PS and functions in tissue morphogenesis and movement during *Drosophila* development. *Development* (in the press)). □

Methods

Cloning, mutagenesis and transgenic animals. Genomic sequences flanking the *Vol*¹ insertion were isolated by plasmid rescue³⁹. Wild-type genomic clones were isolated from a Canton-S library made in lambda DASHII (provided by M. Eberwine); cDNA clones were isolated from libraries prepared from *Drosophila* head RNA (provided by C. Hall). The 4.6-kb *Vol*-1 RNA sequence is represented by a cDNA of ~4,600 residues. The 4.4-kb *Vol*-s RNA is represented by 3,366-bp cDNA. The *Vol*² excision was isolated after dysgenesis. Briefly, flies carrying the *Vol*¹ enhancer detector element were crossed to *X*^{cs}; *CyO*/2^{cs}; *ry* Sb P[*ry*⁺, Δ 2-3,99B]/TM6, *Tb* ('cs' denotes chromosomes derived from a wild-type Canton-S stock). Dysgenic progeny carrying *CyO* were crossed to *X*^{cs}; *CyO*/leo¹³⁷⁵; *ry*^{506-iso} flies. *CyO*; *ry*^{506-iso} progeny were selected for stocks; *ry*^{506-iso} is an isogenic *ry*⁵⁰⁶ chromosome. Excision derivatives were characterized by Southern blotting, extensive PCR analyses, and sequencing of PCR products that cross deletion break points. Because of the nonspecific behavioural effects of mini-white vectors^{40,41}, a new P-factor vector (pCy-20-dhsp) for driving genes behind the hsp70 promoter was constructed with *ry*⁺ as the selectable marker (provided by G. Roman). This vector,

containing an *MluI*–*KpnI* fragment of the *Vol*-s cDNA was injected into *Vol*² embryos. Chromosomal localization of the transgenes and the generation of homozygotes for the transgenes were performed by standard crosses. The presence of the *Vol*² allele in the transgenic animals was confirmed by PCR analyses of genomic DNA. The *Vol* transgene resides on the X and 2nd chromosome, respectively, in VS-T2 and VS-T3. Flies were collected in clean food vials, transferred to pre-warmed food vials, and immersed in a water bath at 37°C for 15 min. Following heat-shock, flies were transferred to room-temperature food vials and stored until testing.

RNA blots and RT-PCR analyses. For RNA blots, poly(A)⁺ RNA was isolated after tissue homogenization in guanidinium-isothiocyanate, banding in CsCl gradients, and by batch adsorption to oligo-(dT) cellulose. Poly(A)⁺ RNA was fractionated (10 µg per lane) by formaldehyde–agarose gel electrophoresis. For RT-PCR experiments, total RNA from heads or whole flies was extracted using Trizol (Gibco-BRL) according to the manufacturer's instructions. Each RT reaction contained 3 µg total RNA, 500 ng oligo-(dT), and 200 U SuperScript II (Gibco-BRL) in a total volume of 20 µl. The reactions were incubated at 42°C for 50 min and digested with 10 U *Sau3AI* and 10 U *AclI* at 37°C for 3 h. RNase A treatments (10 µg) prior to RT reactions were for 1 h at 37°C. Portions of 0.2–5.0% of the RT reactions were amplified using PCR for 20 cycles. For amplification of *Vol* first-strand cDNAs, an antisense primer than anneals to the common 2nd exon of *Vol* (857 nucleotides 3' of the translation start site) was used in combination with sense primers specific for the first exon of either *Vol*-l or *Vol*-s (84 and 118 nucleotides 5' to translation start site, respectively). For amplification of PKA, primers that anneal to the 2nd exon of *DCO* were used. PCR products (942, 975 and 356 bp for *Vol*-l, *Vol*-s and PKA, respectively) were electrophoresed in agarose gels, blotted and hybridized to ³²P-labelled probes.

Histology, generation of antisera, and immunoblotting. β-Galactosidase staining and haematoxylin and eosin staining were performed as described^{21,22,29–31}. For generation of antisera, rabbits were injected with a purified glutathione S-transferase (GST)–*Vol* fusion protein containing either *Vol* amino-acid sequence 1087–1115 (C terminus) or 358–496 (extracellular domain). For immunohistochemistry using anti-*Vol* antisera and the anti-FasII monoclonal antibody 1D4, adult heads were fixed in 4% paraformaldehyde at 4°C for 2 h and incubated in 25% sucrose in Ringer's solution at 4°C overnight. Serial cryosections (10 µm) were incubated with affinity-purified anti-*Vol* or anti-FasII antibody at 4°C overnight. For anti-D-Mef2 and anti-Leonardo staining, adult heads were fixed in Carnoy's solution for 4 h, embedded in paraffin, sectioned and incubated with the appropriate antiserum overnight at 23°C. In all cases, the antigen–antibody complexes were visualized using the Elite Vectastain ABC kit (Vector Laboratories). For immunoblotting, protein extracts were prepared by homogenizing whole flies in 2× Laemli's sample buffer containing 1% β-mercaptoethanol at 75°C for 30 min. Fly extracts (0.5 fly equivalents per lane) were electrophoresed on SDS–polyacrylamide gels and blotted onto PVDF membranes (Millipore). Blots were incubated with affinity-purified anti-*Vol* sera overnight at 4°C, HRP-conjugated goat-anti-rabbit IgG (Jackson Laboratories) for 1 h at 23°C, and visualized with SuperSignal Chemiluminescent substrate (Pierce).

Behavioural analyses. The differential olfactory conditioning method⁴, pairing the presentation of one odour with aversive shock and a second odour with the absence of shock, was used to assess learning and memory performance. Training and testing were performed blind to strain under dim red light at 23–25°C and 63–68% relative humidity using procedures described²². In each group, a performance index was calculated as the fraction of flies that avoided the CS+ minus the fraction of flies that avoided the CS–, and multiplied by 100. In practice, performance-index scores ranged from 0 (naive behaviour) to 100 (perfect performance). Because the minimum possible time between training and testing is 3 min (owing to handling and recovery of flies after transfer), 3-min memory reflects the earliest testable time point. To test longer-term memory, the flies were returned as a group to their collection vials for the appropriate retention interval and then tested as above. Odour avoidance was calculated as the fraction of flies that avoided the odour in one arm minus the fraction of flies that avoided fresh air provided in the control arm, and multiplied by 100. Electroshock avoidance was calculated similarly.

Statistics. Statistical analyses were performed using Statview 2.0 (Abacus Concepts, Berkeley, CA). Overall analyses of variance (ANOVA) were followed by planned comparisons contrasting the relevant groups. Error rate arising

from multiple comparisons was controlled by dividing the alpha level by the number of comparisons being performed on a given set of data⁴². Only statistical results pertinent to the discussion of result are presented in the text; the complete statistical analysis is available as Supplementary Information.

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Detection of intergalactic red-giant-branch stars in the Virgo cluster

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It has been suspected for nearly 50 years that galaxy clusters contain a population of intergalactic stars ripped from the galaxies during cluster formation, or when the galactic orbits pass through the cluster centre^{1–3}. Observational support for the existence of such a stellar population is provided both by measurements of the diffuse light in clusters^{4–9}, and by the recent detection of planetary nebulae with positions or velocities far removed from any observable cluster galaxy^{10,11}. But estimates for the mass of the diffuse population and its distribution relative to the cluster galaxies are still highly uncertain. Here we report the direct detection of intergalactic stars in deep images of a blank field in the Virgo cluster. The data suggest that these stars form approximately one-tenth of the total stellar mass of the cluster. We observe a relatively homogeneous distribution of stars, with evidence of a slight gradient towards M87.

The process of cluster formation undoubtedly involved interactions and mergers of galaxies, and tidal ablation of stars due to the mean gravitational shear of the cluster². Further stripping is likely to have occurred due to 'galaxy harassment', the cumulative effect of many close, high-speed encounters with massive cluster galaxies³. Theoretical predictions of the fraction of mass in the intergalactic population range from 10% to 70%. This large uncertainty stems at a fundamental level from our poor knowledge of when galaxies formed, when and how clusters collapsed, and how the dark matter and baryonic matter were distributed within proto-galaxies^{2,12,13}. Detection and mapping of intergalactic stars could be an important tool for investigating these issues.

Searches for the diffuse light from intergalactic stars are extremely difficult owing to the stringent requirements on background subtraction. Reported detections and upper limits range from 10% to 45% of the total cluster light in various clusters^{6–9}. Even when a signal is detected, it is unclear how much is due to a true diffuse component, and how much to the extension of the galaxy luminosity function below the limits of individual source detection. Intergalactic planetary nebula candidates have been identified in the Virgo cluster (3) and the Fornax cluster (10), but these numbers are not yet sufficient to draw definite conclusions about the distribution of intergalactic stars.

In May 1996 we used the Hubble Space Telescope WFPC2 camera with the F814W (approximately I-band) filter to observe a blank field in the Virgo cluster (Fig. 1). At faint limits compact galaxies are essentially indistinguishable from stars, so the Hubble Deep Field (HDF), which was taken in an area of sky with no foreground cluster, was used as a control. For the comparisons, the HDF images were divided into two groups, each consisting of exposures taken at three pointing positions with total exposure times identical to (within 1%) the Virgo cluster field. Because the sky noise was considerably lower in the HDF images, additional noise was added to match the statistics in the Virgo cluster field. These images were re-reduced and resampled to match the Virgo cluster images.

Figure 2 shows the comparison of source counts for the different images. Fainter than I-band magnitude $I = 26.5$, there is a clear

excess in source counts in the Virgo cluster image. The excess amounts to ~ 630 extra sources to $I = 27.9$. We expect fewer than 20 Galactic foreground stars to this depth. The major uncertainty is the field-to-field variance in galaxy counts, because galaxies at the limiting depth of our image are only marginally resolved. For a square field of angular size θ on a side, the expected variance in the number counts is $\Delta N^2 = N + 2.24N^2A\theta^{1-\gamma}$ where N is the mean number per field, and the angular correlation function follows the power law $w(\theta) = A\theta^{1-\gamma}$ (ref. 14). Using N from the HDF, and adopting $A = 1.5 \times 10^{-4}$ and $\gamma = 1.8$ (ref. 15), the expected standard deviation in galaxy counts is $\Delta N = 80$, well below the 630 excess sources we detect. As a check that the HDF is not highly unusual, source detection and aperture photometry were carried out on an additional control field, centred on the radio galaxy 3C210. Source counts in this field match the HDF to within 20% down to the completeness limit ($I = 27.5$) of the 3C210 field.

The total flux from the 630 excess sources detected in the image of the Virgo cluster corresponds to a total magnitude $I = 20.53$, or a surface brightness (dividing by the area of the field) of $\mu_I = 31.16$ mag per square arcsec. The source density is relatively uniform across the field, with a marginally significant gradient towards M87. To estimate the surface brightness and total mass of stars below our detection limit, consider a population with a metallicity, expressed as a decimal logarithm relative to the solar iron-to-hydrogen ratio, $[Fe/H] = -0.7$, an age of 13 Gyr, a Salpeter³² initial mass function, and a distance of 18.2 Mpc. According to the theoretical luminosity function¹⁶, stars brighter than $M_I = -3.4$ (corresponding to a limiting magnitude $I = 27.9$ for the assumed distance) contribute 16% of the total flux from the stellar population.

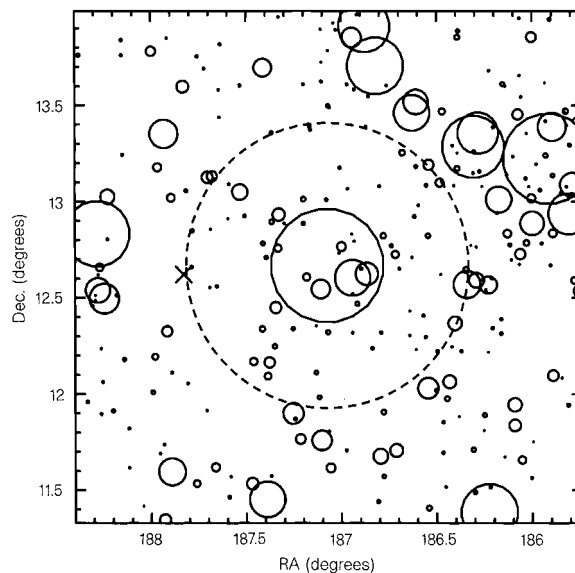


Figure 1 The 'X' marks the position of the HST images in this view of the core of the Virgo cluster. The solid circles mark positions of cluster galaxies, and are scaled by the square root of the galaxy luminosity. The central bright galaxy is M87, and the dashed circle marks a region 44.5 arcmin from M87. The field is located on this circle ~ 235 kpc east of M87 (J2000 coordinates; right ascension (RA) 12 h 33 min 52 s, declination 12° 21' 40") and well separated from any of the other bright galaxies in the cluster. The WFPC2 field covers 5.33 square arcmin. The nearest galaxy to the WFPC field is a dE galaxy with a magnitude $B = 18.0$, 6.6 arcmin away. The nearest bright galaxy is NGC4552 ($B = 10.8$), at a distance of 23 arcmin (~ 122 kpc if the distance to the Virgo cluster is 18.2 Mpc). The total exposure time for the observation was 33,500 seconds. The exposures were taken at three positions separated by ± 0.33 arcsec to improve resolution and aid in removing hot pixels and other cosmetic defects. Data were reduced and images combined following the procedures for the Hubble Deep Field (HDF)³¹. The final reduced images were resampled using the 'drizzle' code (A. Fruchter and R. Hook, manuscript in preparation) onto a pixel grid of 0.08 arcsec.

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Our detection of 630 sources thus implies an underlying surface brightness of $\mu_I = 29.14$. The theoretical colour of the population is $B - I = 1.97$ where B is the B-band magnitude, so the B-band surface brightness would be $\mu_B = 31.33$; the surface mass density would be $0.14 M_\odot \text{pc}^{-2}$ if the initial mass function continues to $0.1 M_\odot$ (here $M_\odot$ is the solar mass).

An empirical estimate of the surface brightness can be made by comparison to previous HST observations of the galaxy NGC3379 (ref. 17). In those observations, $\sim 39,000$ stars were detected in the three Wide Field detectors, of which we estimate 5,000 ($\pm 50\%$) were brighter than $I = 26.9$ (roughly equivalent to our detection limit in Virgo; see below). The NGC3379 surface brightness at the radius of these observations is $\mu_B = 27.3$ (ref. 18). The difference in distance moduli between NGC3379 and the Virgo Cluster is 1.0 mag (ref. 19). Thus our detection of 630 stars in Virgo implies a surface brightness $-2.5 \log(630/5,000) + 1.0$ magnitudes fainter, or $\mu_B = 30.6$, in reasonable agreement with the theoretical determination, given the uncertainties.

The intergalactic stars are likely to have originated primarily from the elliptical and S0 galaxies in the cluster. The early-type galaxies are more numerous in the cluster, have older stellar populations (and hence higher stellar mass-to-light ratios), and are likely to have inhabited the central magaparsec of the cluster for much longer than the spiral and irregular galaxies²⁰. To compare the intergalactic light to the light emerging from the early-type galaxies, we divide the Virgo cluster catalogue²¹ into successively larger annuli of 70 galaxies centred on M87; we then determine mean surface brightness versus radius, where the mean surface brightness is the total flux from galaxies divided by the area of each annulus. This gives $\mu_B = 28.7$ at radius $r = 44.5$ arcmin. (This becomes $\mu_B = 27.9$ if spirals and irregulars are included.) The implication is that $\sim 10\%$ of the stellar mass in the cluster is in the intergalactic component.

Although the distinction between intracluster stars and the M87 halo becomes purely semantic at a radius of 235 kpc, it is of some interest to compare the inferred surface brightness to the extrapolated surface brightness of M87. An $r^{1/4}$ -law fit to the Caon *et al.*²² photometry between 4.2 and 9.2 arcmin, predicts a surface bright-

ness at $r = 44.5$ arcmin of $\mu_B = 30.3$ along the major axis, and 33.4 along the minor axis. Our field is situated 25° from the minor axis. Thus our detected number of stars is higher than expected from a pure extrapolation of the visible portion of M87. NGC4552, located 28.8 arcmin from our field, could also contribute to the source counts. Even if we assume no tidal truncation, a fit to the minor-axis photometry from 4.3 to 6.5 arcmin predicts $\mu_B = 32.5$ at the position of our field, which would correspond to $\sim 25\%$ of the detected source density. Overlapping haloes of galaxies evidently account for only a portion of the stars we detect, unless the outer profiles deviate substantially from the inner $r^{1/4}$ laws.

The estimated stellar mass fraction is sensitive to the age and metallicity of the population. For a distance modulus $(m - M)_0 = 31.3$, metallicity $[\text{Fe}/\text{H}] < -0.4$ and age > 1 Gyr, the underlying stellar mass fraction for our sample can plausibly range from 4% to 12%. At still younger ages the mass fraction goes down, while at still higher metallicities it goes up. Recent measurements of the Virgo cluster distance modulus range from $(m - M)_0 = 31.0$ to 31.7 (refs 19, 23, 24). The mass fraction is still $\sim 5\text{--}10\%$ unless the distance modulus is at the high end of this range and the metallicity is greater than $[\text{Fe}/\text{H}] = -0.7$.

The present observations thus favour a rather small mass fraction in the diffuse population. In contrast, Theuns and Warren¹¹ infer—from the detection of 10 planetary nebula candidates—that 40% of the stellar mass is in the diffuse population of the Fornax cluster. Our results are consistent only if the intergalactic population is metal-rich, or the Virgo cluster is more distant than ~ 20 Mpc. However, the planetary-nebula estimate suffers from small number statistics, possible contamination of the sample by background emission-like galaxies, and variations in the planetary-nebula specific frequency with stellar population.

For an assumed distance of 18.2 Mpc, the total gravitating mass estimated from X-ray observations in the central 240 kpc of the Virgo cluster is $(1.2\text{--}3.5) \times 10^{13} M_\odot$ (ref. 25), and the corresponding mass-to-light ratio $M/L_B \approx 260$. Most of the light in the central region of the Virgo cluster resides in the central 10 arcmin of M87, while most of the mass resides at larger radii. For the region outside this central 10 arcmin, our observations imply a mean $M/L_B \approx 700$. The relatively smooth distribution of mass inferred from the X-ray observations²⁵ suggests that most of the intergalactic material was stripped by tidal interactions with the cluster potential, although we cannot rule out the possibility that some of the stars formed *in situ*, or that some were stripped off by impulsive interactions between galaxies ('harassment').

The luminosity of the tip of the red giant branch has been promoted as a distance indicator of comparable precision to Cepheid variables²⁶. Although the number of stars in our sample is not large enough for a precise distance estimate, the shape of the observed luminosity function is consistent with that seen in the halo of NGC5128 (ref. 27), and in the halo of NGC3379 (ref. 17), shifted to a distance modulus in the range $31.2 < (m - M)_0 < 31.6$.

Future observations should reveal whether the profile of the intergalactic stars is similar that of the gravitating mass, whether it is centred on M87, and whether it is smoothly distributed. The new instruments on the HST make it possible to measure the metallicity distribution of the diffuse stellar population and compare it to the metallicity distribution in the outer parts of elliptical galaxies and in the X-ray-emitting gas. Star counts also make possible the measurement of the tidal radii of galaxies in the Virgo cluster, and searching for the wakes of galaxies moving through the intracluster material. The combination of HST imaging and ground-based detection of planetary nebulae provides a powerful new probe of the cluster environment and of this nearly invisible population of stars. $\square$

Methods

The zero-point of the instrumental magnitude scale was established by measuring the fluxes of the few bright isolated stars in the field of 0.5-arcsec

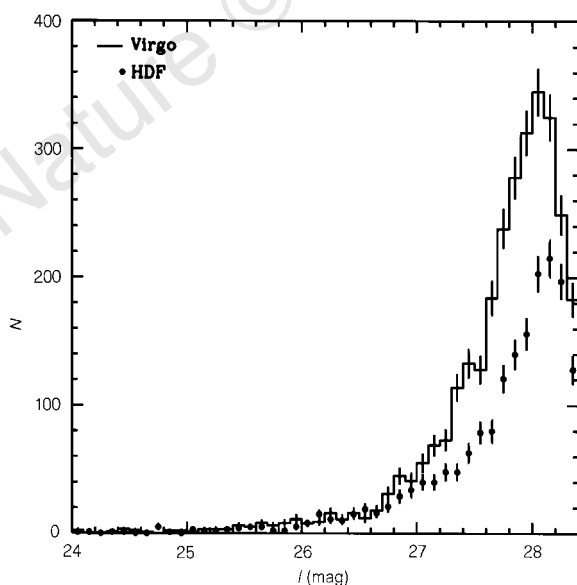


Figure 2 Comparison of source counts in the Virgo cluster field and the Hubble Deep Field (HDF). N is the number of sources per 0.1 mag detected in each field. The histogram is the Virgo cluster field, and the points with error bars are the HDF; magnitudes are based on point-spread function fitting. The excess in the Virgo cluster field begins at I-band magnitude $I \approx 26.8$. Source detection was performed using the program DAOFIND, and profile-fitting photometry was performed with the IRAF implementation of the DAOPHOT ALLSTAR code³³. See Methods for further details.

radius apertures following the standard prescription²⁸. Additional corrections were made as follows: (1) +0.05 mag to correct for the so-called 'long exposure' effect²⁹; (2) -0.04 mag, which is the appropriate colour term to convert to standard Cousins's *I* magnitudes for red stars with $V - I \approx 1.5$ (ref. 28); and (3) -0.04 mag to allow for the expected foreground extinction³⁰. We estimate that the combined uncertainty from the zero-point calibration and the various correction terms amounts to 0.06 mag. Clearly extended sources were removed from the catalogue by restricting our analysis to sources with a DAOPHOT sharpness parameter $-0.6 < s < 0.4$. A similar procedure was also followed for the HDF control field. Finally, we created 12 test data sets in which 265 simulated stars were added to the real data frames. The input magnitudes of the simulated stars covered the range from 23.5 to nearly 30. We then processed these frames in the same way as the original data, and produced a matrix relating the recovered magnitudes and detection efficiency to the input magnitudes of the simulated stars. From these simulations we estimate that the catalogue is $\sim 80\%$ complete at $I = 27.9$, and use this as the limiting magnitude in our analysis.

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Phase matching using an isotropic nonlinear optical material

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Frequency conversion in nonlinear optical crystals^{1,2} is an effective means of generating coherent light at frequencies where lasers perform poorly or are unavailable. For efficient conversion, it is necessary to compensate for optical dispersion, which results in different phase velocities for light of different frequencies. In anisotropic birefringent crystals such as LiNbO₃ or KH₂PO₄ ('KDP'), phase matching can be achieved between electromagnetic waves having different polarizations. But this is not possible for optically isotropic materials, and as a result, cubic materials such as GaAs (which otherwise have attractive nonlinear optical properties) have been little exploited for frequency conversion applications. Quasi-phase-matching schemes^{1,3}, which have achieved considerable success in LiNbO₃ (ref. 4), provide a route to circumventing this problem^{5,6}, but the difficulty of producing the required pattern of nonlinear properties in isotropic materials, particularly semiconductors, has limited the practical utility of such approaches. Here we demonstrate a different route to phase matching—based on a concept proposed by Van der Ziel 22 years ago⁷—which exploits the artificial birefringence of multilayer composites of GaAs and oxidised AlAs. As GaAs is the material of choice for semiconductor lasers, such optical sources could be integrated in the core of frequency converters based on these composite structures.

Optical frequency conversion^{1,2} by second-order nonlinear interaction is a way to obtain coherent light in various spectral regions. The frequency doubling process is used, for instance, to obtain green light from the very efficient near-infrared YAG laser, whereas difference frequency generation (DFG) is the basic process for high-power mid-infrared sources such as optical parameter oscillators. The best known nonlinear materials used for frequency conversion are KDP or LiNbO₃ (ref. 2), but several other crystals can be used; for example, KTP (KTiOPO₄), AgGaSe₂, GaAs, and synthetic materials as organic molecules or semiconductor quantum wells⁸. Among the different characteristics of nonlinear crystals, two are of paramount importance. (1) The nonlinear coefficient $\chi^{(2)}$, which is a measure of the strength of the nonlinear interaction, and is related to the degree of asymmetry of the electronic potential at the microscopic level. (2) The ability to match the phase velocities between the different frequencies¹: owing to the optical dispersion of the nonlinear materials, the different waves do not travel at the same velocity in the material. After a distance called the 'coherence length', the nonlinear polarization and the generated wave acquire a phase lag of π , which produces a destructive interference. The effective conversion length is thus limited to the coherence length, which is often as small as a few micrometres. In reciprocal space, the phase-matching conditions expresses the photon momentum conservation.

To increase the coherence length, phase matching can be obtained in birefringent crystals as KDP or LiNbO₃; the polarizations of the different waves are carefully chosen in order to adjust their different phase velocities. GaAs is a very interesting material for nonlinear frequency conversion, because its huge nonlinear coefficient ($\chi^{(2)} \approx 240 \text{ pm V}^{-1}$) gives about one order of magnitude greater efficiency than the commonly used materials. However, as it is an optically isotropic (cubic) semiconductor, it is non-birefringent and

phase matching is impossible. To use this highly nonlinear material in spite of the problem of phase matching, quasi-phase-matching^{1,3} has been used. The sign of the nonlinear interaction is changed periodically by reversing the orientation of the material, in order to compensate for the periodic destructive interference due to phase mismatch. This technique has produced impressive results for LiNbO₃ (ref. 4), but although quasi-phase-matching has been shown in GaAs for both second-harmonic⁵ and difference-frequency⁶ generations, patterning the nonlinear susceptibilities is much more difficult in semiconductors. The technological steps necessary to obtain the material are difficult and the final materials suffer from detrimental losses.

Here we demonstrate perfect phase matching using an isotropic material as a microscopic nonlinear source. Phase matching is obtained by using a built-in artificial birefringence in a new composite multilayer material: the isotropy of bulk GaAs is broken by inserting thin oxidized AlAs ('Alox') layers in GaAs. The resulting composite material is not isotropic, and this is used to get birefringent phase matching, but the only nonlinear material present in the structure is the isotropic GaAs. The use of this so-called form birefringence^{9,10} for nonlinear frequency conversion was proposed in 1975 by Van der Ziel⁷. Since then, however, the experimental realization of this proposal has not been achieved, because it has not been possible to find a well suited couple of materials having a high nonlinear coefficient and a high enough refractive index contrast for form birefringence phase matching.

A first, intuitive, way to understand the origin of form birefringence is to consider the macroscopic crystal formed by a GaAs/Alox multilayer system. GaAs is a cubic semiconductor of point group $\bar{4}3m$, and is therefore non-birefringent. The presence of thin Alox layers grown on a (100) substrate breaks the symmetry of three-fold rotation axes and the point group of the composite material is now

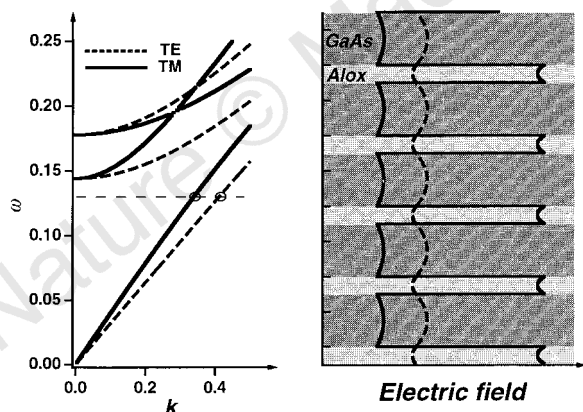


Figure 1 Dispersion relation for an in-plane propagation in a periodic composite material of period d . The material consists of 25% of Alox (refractive index $n \approx 1.6$; see text) and 75% of GaAs ($n \approx 3.5$), for TM modes (full line) and TE modes (dotted lines). The physical origin of form birefringence appears in the mode wavefunction, pictured on the right for a frequency $\omega = 0.13(2\pi/d)$ (corresponding to the open circles in the dispersion relation). These Bloch waves have been calculated using standard periodic multilayer theory¹⁰. The direction of propagation is perpendicular to the plane of the figure. Due to the continuity of the electric displacement ϵE normal to the layers, the TM mode (solid line in both panels) has a significant overlap with the low- ϵ layer (Alox, shown in light grey in the right panel), and a lower average dielectric constant. The continuous TE electric field (dotted line in both panels) has a higher value in GaAs (dark grey, right panel), and a higher average dielectric constant.

$\bar{4}2m$, the same as KDP. This artificial material has the nonlinear properties of GaAs (in particular, the same tensorial character and roughly the same nonlinear coefficient, if we neglect the small reduction of the average nonlinear susceptibility due to the zero contribution of the thin Alox layers), but the linear optical symmetry of KDP. This is possible by taking advantage of the microscopic nature of the nonlinear polarization in GaAs and the macroscopic engineering of the refractive index on the scale of the extended electromagnetic wavelengths. We note that with (111) orientated GaAs, the introduction of Alox layers switches the point group from $\bar{4}3m$ to $3m$, which is that of another nonlinear material, LiNbO₃; the same birefringence properties are of course expected.

Van der Ziel⁷ calculated form birefringence in the multilayer system directly from Maxwell's equations. Another physical explanation can be given using modal wavefunction considerations. We consider a periodic multilayer material, with light propagating in the plane of the layers. Following Joannopoulos¹¹, for a given wavevector the frequency of an allowed electromagnetic mode in a composite medium increases with the fraction of electric field in the low-index material. The difference between the transverse electric (TE) and transverse magnetic (TM) polarizations then arises from the continuity equations at the boundaries between the two materials, as illustrated in Fig. 1. In the TM polarization, the continuity of the electric displacement forces the electric field to have a large value in the low-index material; this mode therefore has a higher frequency ω for a given wavevector k . For a large wavelength compared to the unit cell, the light experiences an effective medium, that is, a homogeneous-like material, the inhomogeneities of the dielectric constant being averaged on the wavelength scale. This is why the dispersion relation $\omega(k)$ in Fig. 1 is linear at the origin. Form birefringence then appears as the difference between the slopes of the dispersion relations for TE and TM waves. In this long-wavelength

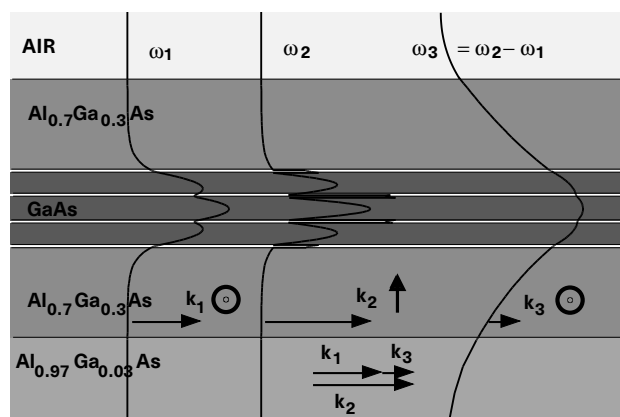


Figure 2 Difference frequency generation (DFG) process in the sample. Here ω_1 and ω_2 are the pump frequencies of wavelengths 1.32 μm and 1.058 μm , respectively, and ω_3 is the frequency difference of wavelength 5.3 μm . Three periods of the composite material GaAs (325 nm)/Alox (40 nm) constitute the core of the waveguide. The birefringence of the composite material was engineered to compensate for the dispersion arising from both the natural dispersion in bulk GaAs and the optical confinement dispersion in the waveguide. The sample was grown by molecular beam epitaxy on a GaAs (100) substrate and consists of: 2,800 nm Al_{0.97}Ga_{0.03}As; 1,500 nm Al_{0.70}Ga_{0.30}As (waveguide cladding layers), three periods of birefringent composite material (40 nm Alox; 325 nm GaAs) $\times$ 3 and 40 nm Alox; 1,500 nm Al_{0.70}Ga_{0.30}As and a final 30-nm GaAs cap layer. The oxidation process is described in detail in ref. 15. The three modes involved in the DFG process are pictured together with their polarization ($\uparrow$ for TM, $\odot$ for TE). The higher overlap of the TM mode with the low-refractive-index Alox layers is apparent, which is the origin of form birefringence. The arrows indicate the "phase matching" momentum conservation. $\mathbf{k}_1 + \mathbf{k}_3 = \mathbf{k}_2$, where $\mathbf{k}_i$ indicates the wavevector of the wavenumber i .

approximation, the two dielectric constants of the uniaxial composite material are given by:

$$\epsilon_{TE} = \alpha_1 \epsilon_1 + \alpha_2 \epsilon_2 \quad (1)$$

$$\frac{1}{\epsilon_{TM}} = \frac{\alpha_1}{\epsilon_1} + \frac{\alpha_2}{\epsilon_2} \quad (2)$$

where α_i and ϵ_i are the filling factors ($\alpha_1 + \alpha_2 = 1$) and the dielectric constant, respectively, of the two constitutive materials 1 and 2. These equations are analogous to electrical series and parallel capacitors. This is not surprising, because the charge equality $C_1 V_1 = C_2 V_2$ between series capacitors 1 and 2 is nothing but the static limit of the electric displacement continuity relation for TM waves $\epsilon_1 E_1 = \epsilon_2 E_2$, and the bias equality $V_1 = V_2$ for parallel capacitors 1 and 2 is equivalent to the electric-field continuity for TE waves $E_1 = E_2$. (Here C represents capacitance, and V voltage.)

It appears from equations (1) and (2) that form birefringence ($\sqrt{\epsilon_{TE}} - \sqrt{\epsilon_{TM}}$) increases with the refractive-index contrast between the two materials in the multilayer, as do photonic bandgap effects in photonic crystals¹¹. Although form birefringence in a GaAs/AlGaAs multilayer structure has been proposed for phase matching⁷, the refractive-index contrast between GaAs ($n \approx 3.5$) and AlAs ($n \approx 2.9$) is too low to provide the birefringence required to compensate for the dispersion. This is why we have used thin-film layers of Alox ($n \approx 1.6$) in GaAs to get sufficient form birefringence. Alox results from selective oxidation (at 400–500 °C in a water-vapour atmosphere) of AlAs layers embedded in GaAs. The technology of AlAs oxidation emerged¹² in the early 1990s; since then Alox has led to significant advances in the field of semiconductor lasers¹³ and Bragg mirrors¹⁴ thanks to its refractive index contrast with GaAs.

We have demonstrated phase matching of a DFG process in a waveguide containing four Alox layers. The structure which was used for the DFG is shown in detail in Fig. 2, together with the three modes involved in the nonlinear interaction.

We first characterized the form birefringence of the sample. The details of this experiment, using surface emitting second-harmonic generation, are given in ref. 15. A form birefringence $n_{TE} - n_{TM} = 0.154$ was measured. Even higher birefringences of the order of 0.2 have been obtained with different samples. This birefringence is sufficient to phase-match mid-infrared generation between 3 μm and 10 μm by DFG. We note that by increasing the width of Alox layers (as in the example of Fig. 1), much higher

birefringences up to 0.65 could be achieved, but this is not necessary here.

For the DFG experiment, two continuous-wave pump lasers (a Nd:YAG with wavelength $\lambda = 1.32 \mu\text{m}$, and a tunable Titanium: Sapphire (Ti:Sa) were end-fire coupled in a 4- μm -wide, 1.2-mm-long channel waveguide. Using the $\chi_{xyz}^{(2)}$ element of GaAs, the DFG process was:

$$(1.058 \mu\text{m}, \text{TM}) - (1.32 \mu\text{m}, \text{TE}) \rightarrow (5.3 \mu\text{m}, \text{TE}) \quad (3)$$

as it appears in Fig. 2. The infrared signal is shown in Fig. 3 as a function of the Ti:Sa wavelength. This function has the well known $((\sin x)/x)^2$ shape, which is clear evidence of phase matching. All the polarization selection rules for equation (3) have been checked. These experiments show that perfect phase matching has been achieved with a cubic nonlinear material.

A mid-infrared output power of 80 nW was obtained for 0.4 mW and 11.6 mW of Nd:YAG and Ti:Sa pump powers, respectively. This result can easily be pushed into the microwatt range by increasing pump powers and reducing scattering losses originating from processing; such power levels are interesting for mid-infrared spectroscopic applications. We note also that the generated wavelength (5.3 μm) is in the absorption range of LiNbO₃, where few nonlinear optical materials exist. Fourier-transform infrared spectroscopic measurements show that absorption losses in Alox start for wavelengths $>7.5 \mu\text{m}$, opening a very large spectral range for tunable DFG. The efficiency of this frequency converter is limited by the mid-infrared losses which are essentially due to scattering on the ridge inhomogeneities introduced during the technological process. Improving the technological process to reduce these losses will be very important for the success of this composite material in frequency conversion devices. The tunability of the mid-infrared wavelength was demonstrated by varying the temperature. We have measured a linear dependence of the signal wavelength from 5.2 to 5.6 μm with a temperature scan from 0 to 150 °C (Fig. 3 inset). No degradation of the sample was observed during temperature cycles.

The birefringence of the composite structure is sufficient not only to phase match DFG in the mid-infrared, but also for second-harmonic generation around 1.55 μm . We give these two examples because of their interest for applications: continuously tunable mid-infrared compact sources are desirable for pollutant detection in the molecular fingerprint region or for process monitoring, and a 1.55- μm signal can be shifted by mixing it with a 0.75- μm pump. The latter function is required in wavelength division multiplexing systems. Other possible implications go beyond use of the simple passive nonlinear material: GaAs is also the favoured material for quantum-well lasers, and the possibility of integrating quantum wells in the core of the nonlinear waveguide is under study. In a first step, this would enable DFG with only one external source, the other frequency being given by the 'internal' quantum-well laser. In a second step, parametric fluorescence from this laser would make a completely monolithic micro-optical parametric oscillator—on a GaAs chip, tunable with temperature—a realistic possibility. □

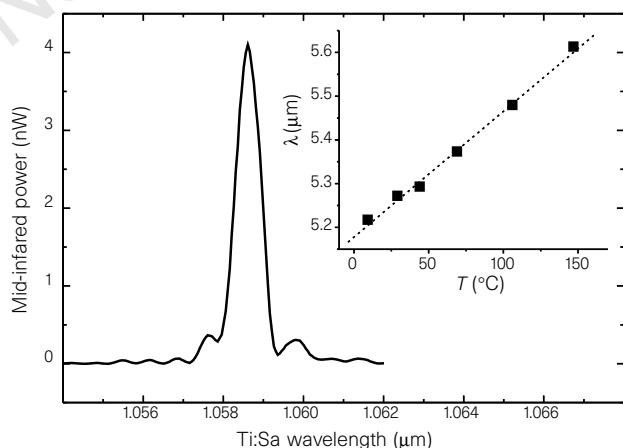


Figure 3 Mid-infrared DFG signal measured by an InSb detector, as a function of the wavelength of the Ti:Sa laser. The pump powers were 0.2 mW and 1.6 mW for the YAG and Ti:Sa lasers, respectively. This function has the well known shape of a phase matching resonance: it is a $((\sin x)/x)^2$ function, characteristic of momentum conservation in a fixed interaction length³. Inset, signal wavelength tunability with temperature.

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Broken symmetry and pseudogaps in ropes of carbon nanotubes

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Since the discovery of carbon nanotubes¹, it has been speculated that these materials should behave like nanoscale wires with unusual electronic properties and exceptional strength. Recently, 'ropes' of close-packed single-wall nanotubes have been synthesized in high yield². The tubes in these ropes are mainly of the (10,10) type³, which is predicted to be metallic^{4–6}. Experiments on individual nanotubes and ropes^{7,8} indicate that these systems indeed have transport properties that qualify them to be viewed as nanoscale quantum wires at low temperature. It has been expected that the close-packing of individual nanotubes into ropes does not change their electronic properties significantly. Here, however, we present first-principles calculations which show that a broken symmetry of the (10,10) tube caused by interactions between tubes in a rope induces a pseudogap of about 0.1 eV at the Fermi level. This pseudogap strongly modifies many of the fundamental electronic properties: we predict a semimetal-like temperature dependence of the electrical conductivity and a finite gap in the infrared absorption spectrum. The existence of both electron and hole charge carriers will lead to

qualitatively different thermopower and Hall-effect behaviours from those expected for a normal metal.

Carbon nanotubes are tubular structures which are typically a few nanometres in diameter and many micrometres in length. These quasi-one-dimensional systems are predicted^{4–6} to have an electronic structure dictated by their geometric structure. In particular, an isolated armchair-type nanotube (one whose roll-up indices are (n, n)) has two linear bands which cross at the Fermi level. These two linear bands give rise to a constant density of states near the Fermi level and to metallic behaviour in conduction and other physical properties. One of the bands has π -bonding and the other has π -antibonding character. An isolated (n, n) nanotube has n mirror planes containing the tube axis. The π -bonding state is even (the wavefunction has no sign change) and the π -antibonding state is odd (sign change) under these symmetry operations. The band crossing is allowed and the armchair nanotube is metallic as shown schematically in Fig. 1a. It is precisely this symmetry of the isolated (n, n) tube that gives its desired metallic behaviour⁹, and this property motivates many of the recent electrical measurements. Breaking of this symmetry, however, will completely alter this picture.

With the rope axis vertical, if one were to separate the tubes in a rope far enough to eliminate any interactions between the tubes, then the energy bands of the rope would have no dispersion in the horizontal plane, and the band structure along any vertical line in reciprocal space would be identical to that of a single isolated tube, with a band crossing as shown in Fig. 1a. However, the actual distances between tubes in the ropes are small enough that each nanotube can feel the potential due to all the other nanotubes. As a result of this perturbation, the Hamiltonian at any point k where the two bands used to cross becomes

$$H_k = \begin{pmatrix} \epsilon_0 + \delta_{11} & \delta_{12} \\ \delta_{21} & \epsilon_0 + \delta_{22} \end{pmatrix} \quad (1)$$

where ϵ_0 is the unperturbed energy. The diagonal matrix elements δ_{11} and δ_{22} merely act to shift the energy and location in k -space of the band crossing. It is the off-diagonal elements that cause quantum-mechanical level repulsion and therefore open a gap as shown schematically in Fig. 1b. If the vertical line through k has high

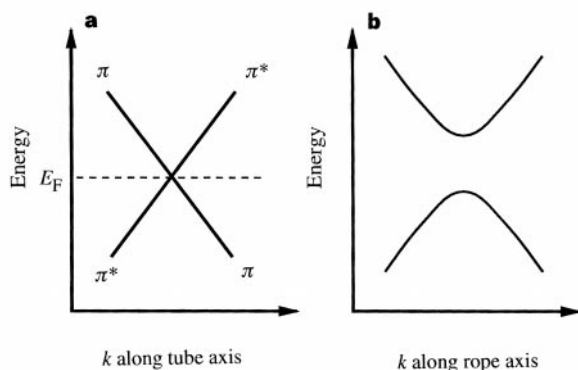


Figure 1 Band crossing and band repulsion. **a**, Schematic diagram of the crossing of two linear bands for an isolated (n, n) carbon nanotube. One band has π -bonding character and the other has π -antibonding (π^*) character. E_F is the Fermi energy and k is the wavevector. **b**, Repulsion of bands due to breaking of mirror symmetry.

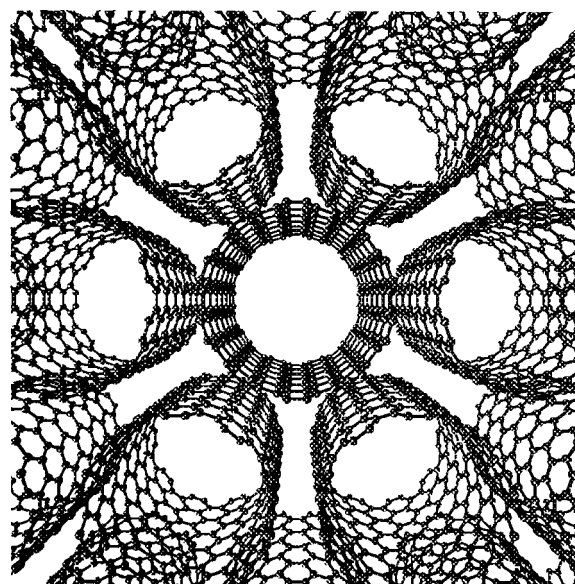


Figure 2 Perspective view of a rope of (10,10) carbon nanotubes. Along the horizontal axis one can see the alignment of the chains of hexagons between neighbouring nanotubes.

symmetry, the off-diagonal matrix elements may still be zero and a crossing may persist. However, at a general k -point the inter-tube interactions, which have not been considered in detail in the past, will dramatically change the physics of the ropes.

To determine the character and magnitude of the pseudogap, we make calculations including the full interaction between the tubes using both the *ab initio* pseudopotential local-density-functional method and the empirical pseudopotential method¹⁰ (EPM). The empirical pseudopotential is generated so as to reproduce the band structure of a single graphene sheet and of graphite¹¹ accurately near the Fermi level. With this choice, our potential captures both the intra- and inter-tube interactions and describes the electronic structure of the rope correctly. With the EPM, the computational effort is considerably reduced, allowing a detailed investigation of the electronic states of the ropes. Our *ab initio* pseudopotential band structures for the ropes at selected k -points confirm the EPM results.

Experimentally, the ropes are found to contain hundreds of tubes of nearly uniform diameter forming a triangular lattice³. In this work, the (10,10) tubes are packed in a hexagonal array in the x - y plane with the tube axes along the z -direction and with the intertube carbon-carbon distance being 3.3 Å. This arrangement is depicted in Fig. 2. The rotational orientation of the tubes relative to each other determines the symmetry of the structure and has an important bearing on the band structure near the Fermi level.

First, we consider the general case where the tubes are rotated by an arbitrary angle (that is misaligned) about the z -axis, so that the rope as a whole loses all vertical mirror planes. The symmetry is low (C_{2h} group) and the off-diagonal matrix element $\delta_{21}(k) = \langle \pi^* | H_k | \pi \rangle \neq 0$ in general. The two bands near the Fermi energy repel, and open a gap as explained above. The rope does not immediately become a semiconductor, however. Because of the periodicity in the x - y plane, different perpendicular components $k_{\perp} = (k_x, k_y)$ of k -vectors exist in the Brillouin zone and the split bands go up and down by different amounts according to the magnitude of the diagonal matrix elements $\delta_{11}(k) = \langle \pi | H_k | \pi \rangle$ and $\delta_{22}(k) = \langle \pi^* | H_k | \pi^* \rangle$. Bands at different $k_{\perp}$ tend to overlap, and so even if there is a gap along the k_z -direction for each $k_{\perp}$, the rope may still be metallic.

To model this general case, we perform a calculation where a vertical row of hexagons in one tube lines up 1.5° off from perfect alignment with a hexagon row in one of the neighbouring tubes. The calculated density of states (DOS) is plotted with a broken line in Fig. 3a. Gaps open in the band structure which create a valley of ~0.1 eV width in the DOS curve. The DOS at the Fermi level is still about 1/3 of the average density of states: this non-zero value is due to the overlap of bands at different $k_{\perp}$ as expected from the above argument. We call this valley in the DOS at the Fermi level a

pseudogap. This is in sharp contrast to the DOS of an isolated tube which is essentially constant within a range of ± 1 eV from the Fermi energy.

To examine the effect of orientation, we next consider the special case where the tubes are aligned so that a vertical chain of hexagons along one tube lines up exactly with another chain of hexagons on a neighbouring tube. This is the most symmetric situation (D_{2h} group), and some reflection symmetry is preserved. However, unlike the case of the isolated tube, band crossing in the three-dimensional rope requires additional conditions on the electronic states. Our calculations show that the crossing of the two linear bands does occur along particular high-symmetry lines of $k_{\perp}$ in the Brillouin zone. Previous calculations on a hexagonal lattice of the smaller (6,6) carbon nanotube have shown similar features¹². At all other values of $k_{\perp}$, bands again split. The calculated DOS in this case is plotted with a solid line in Fig. 3a. As the measure of the high-symmetry k -points in k -space is zero, there is no significant change in the DOS near the Fermi level. This leads to the important conclusion that the DOS (and hence properties related to it) does not depend on the details of the relative orientations of the tubes. The existence of the pseudogap due to the broken symmetry in the rope makes the conductivity and other transport properties significantly different from those of isolated tubes, even without considering the effect of local disorder in low dimensions^{13,14}. The carrier density will increase as the temperature is raised because the DOS increases quickly away from the Fermi level, as shown in Fig. 3a. This structure in the DOS will also make the properties of the rope sensitive to doping.

The π -bonding and π -antibonding states produce two Fermi surfaces. Under the assumption of the highest possible symmetry (D_{2h}) of the rope, we find a pocket of electron-like states in the shape of a rhombic pancake and another pocket of hole-like states in a similar shape. As the periodic structure and the symmetry are disturbed in real ropes, we expect that the Fermi surfaces are smeared out to some degree. A rather complex temperature dependence of thermopower and Hall effects is expected because of the coexistence of electrons and holes as well as the partial smearing out of the Fermi surfaces.

To examine the optical properties, we calculate the joint density of states (JDOS) for both orientations of the tubes in the rope (Fig. 3b). The JDOS is defined by

$$\text{JDOS}(E) = \sum_{o,u} \frac{1}{4\pi^3} \int_{\text{BZ}} d^3k \delta(E_k^u - E_k^o - E) \quad (2)$$

where o and u run over the occupied and unoccupied states respectively, and BZ is the Brillouin zone. For an isolated tube, because of the two linear bands crossing at the Fermi level, the JDOS has a nonzero value at $E = 0$ and remains almost constant up to ~1 eV. In a rope, on the other hand, the band repulsion makes the JDOS at $E = 0$ vanish completely. The band overlap for different $k_{\perp}$ does not change the JDOS because the JDOS measures the transition energy for each given (conserved) k -vector, as defined in equation (2). Although the symmetrically aligned tubes do produce band crossings at some high-symmetry k -points, the measure of those k -points contributing to the JDOS at $E = 0$ is again zero, and the misaligned and aligned tubes do not show significant differences in Fig. 3b. Infrared absorption measurements should show the difference in behaviour between the isolated tubes and the rope. However, we wish to note that the vertical (k -conserving) transitions of electronic states assumed in equation (2) may have to be relaxed in the study of ropes. As a real rope does not have a perfectly periodic structure, k -conservation is only approximately valid. In the extreme case of complete disorder, an infrared experiment would reflect the DOS rather than the JDOS. In the present case, an infrared measurement would show features in between the JDOS prediction of a gap of a few tens of meV and the DOS prediction of a pseudogap.

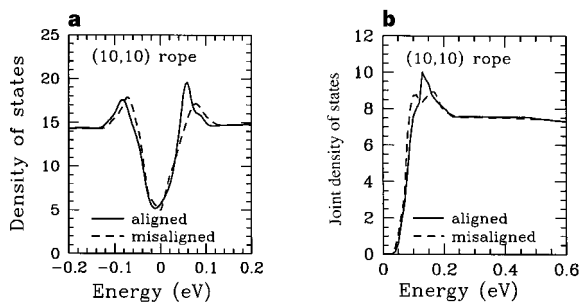


Figure 3 Calculated densities of states. **a**, Calculated density of states for a rope of misaligned (10,10) tubes (broken line) and aligned tubes (solid), in units of states per meV per atom. The Fermi energy is set at zero. **b**, Calculated joint density of states for a rope of misaligned (10,10) tubes (broken line) and aligned tubes (solid), in units of states per meV per atom.

Finally, as a stronger external perturbation in general would lead to a larger band repulsion, a rope could behave like a semiconductor under a sufficiently strong perturbation. This may be an explanation for the quantum dot behaviour of isolated tubes and ropes^{7,8} in which some semiconductor barrier region seemingly develops at the contact where a substantial perturbation in potential is expected to occur. □

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Recognition of the four Watson–Crick base pairs in the DNA minor groove by synthetic ligands

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The design of synthetic ligands that read the information stored in the DNA double helix has been a long-standing goal at the interface of chemistry and biology^{1–5}. Cell-permeable small molecules that target predetermined DNA sequences offer a potential approach for the regulation of gene expression⁶. Oligodeoxynucleotides that recognize the major groove of double-helical DNA via triple-helix formation bind to a broad range of sequences with high affinity and specificity^{3,4}. Although oligonucleotides and their analogues have been shown to interfere with gene expression^{7,8}, the triple-helix approach is limited to recognition of purines and suffers from poor cellular uptake. The subsequent development of pairing rules for minor-groove binding polyamides containing pyrrole (Py) and imidazole (Im) amino acids offers a second code to control sequence specificity^{9–11}. An Im/Py pair distinguishes G–C from C–G and both of these from A–T/T–A base pairs^{9–11}. A Py/Py pair specifies A,T from G,C but does not distinguish A–T from T–A^{9–14}. To break this degeneracy, we have added a new aromatic amino acid, 3-hydroxypyrrole (Hp), to the

repertoire to test for pairings that discriminate A–T from T–A. We find that replacement of a single hydrogen atom with a hydroxy group in a Hp/Py pairing regulates affinity and specificity by an order of magnitude. By incorporation of this third amino acid, hydroxypyrrole–imidazole–pyrrole polyamides form four ring-pairings (Im/Py, Py/Im, Hp/Py and Py/Hp) which distinguish all four Watson–Crick base pairs in the minor groove of DNA.

Because of the degeneracy of the hydrogen-bond donors and acceptors displayed on the edges of the base pairs, the minor groove was thought to lack sufficient information for a complete recognition code¹⁵. But, despite the central placement of the guanine exocyclic N2 amine group in the G,C minor groove^{15,16}, Py/Im and Im/Py pairings distinguish energetically between G–C and C–G^{9–11,17,18}. The neighbouring Py packs an Im to one side of the minor groove resulting in a precisely placed hydrogen bond between Im N3 and guanine N2 for specific recognition^{19,20}. This remarkable sensitivity to single atomic replacement indicates that substitution at the 3 position of one Py within a Py/Py pair can complement small structural differences at the edges of the base pairs in the centre of the minor groove. For A,T base pairs, the hydrogen-bond acceptors at N3 of adenine and O2 of thymine are almost identically placed in the minor groove, making hydrogen-bond discrimination a challenge (Fig. 1)¹⁵. The existence of an asymmetrically placed cleft on the minor groove surface between the thymine O2 and the adenine 2H suggests a possible shape-selective mechanism for A–T recognition²¹. We reasoned that substitution of C3–H by C3–OH within a Py/Py pair would create 3-hydroxypyrrole (Hp)/Py pairings to discriminate T–A from A–T (Fig. 2). Selectivity could potentially arise from steric destabilization of polyamide binding via placement of Hp opposite A or stabilization by specific hydrogen bonds between Hp and T.

Four-ring polyamide subunits, covalently coupled to form eight-ring hairpin structures, bind specifically to 6-base-pair (bp) target sequences at subnanomolar concentrations^{11,18}. We report here the DNA-binding affinities of three eight-ring hairpin polyamides containing pairings of Im/Py, Py/Im opposite G–C, C–G and either Py/Py, Hp/Py or Py/Hp at a common single point opposite T–A and A–T (Fig. 2b). Equilibrium dissociation constants (K_D) for ImImPyPy- γ -ImPyPyPy- β -Dp (1), ImImPyPy- γ -ImHpPyPy- β -Dp (2), and ImImHpPy- γ -ImPyPyPy- β -Dp (3) were determined by

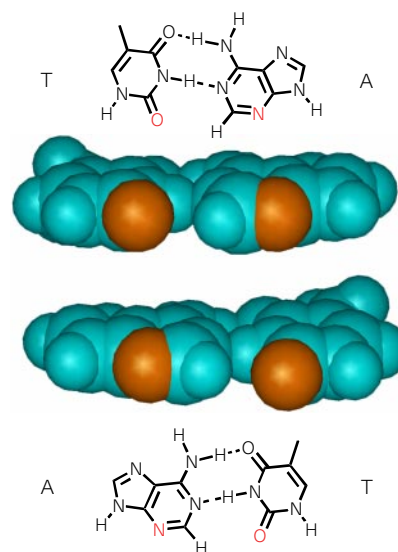


Figure 1 Chemical structures and space-filling models of the T–A and A–T base pairs as viewed from the minor groove of DNA. Models generated using B-form DNA coordinates provided in InsightII. The hydrogen-bond acceptors (N3 of adenine and O2 of thymine) are in red.

Table 1 Equilibrium dissociation constants

	K_D (nM)		
Polyamide*	5'-TGGTCA-3'	5'-TGGACA-3'	$K_{rel}^\dagger$
1 Py/Py	0.077	0.15	2.0
2 Py/Hp	15	0.83	0.06
3 Hp/Py	0.48	37	77

The reported dissociation constants are the average values obtained from three DNase I footprint titration experiments. The standard deviation for each data set is less than 15% of the reported number. Assays were carried out in the presence of 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl₂ and 5 mM CaCl₂ at pH 7.0 and 22 °C.

* Ring pairing opposite T·A and A·T in the fourth position.

† Calculated as $K_D(5'-TGGACA-3')/K_D(5'-TGGTCA-3')$.

quantitative DNase I footprint titration experiments²² on a 3' ³²P-labelled 250-bp DNA fragment containing the target sites, 5'-TGGACA-3' and 5'-TGGTCA-3' which differ by a single A,T base pair in the fourth position (Fig. 3, Table 1).

Based on the pairing rules for polyamide-DNA complexes both of these sequences are a match for control polyamide **1** which places a Py/Py pairing opposite A·T and T·A at both sites. We find that polyamide **1** (Py/Py) binds to 5'-TGGTCA-3' and 5'-TGGACA-3' within a factor of 2 ($K_D = 0.077$ or 0.15 nM respectively). In contrast, polyamide **2** (Py/Hp) binds to 5'-TGGTCA-3' and 5'-TGGACA-3' with dissociation constants that differ by a factor of 18 ($K_D = 15$ nM and 0.83 nM respectively). If the pairing is reversed in polyamide **3** (Hp/Py) the dissociation constants differ again in the opposite direction by a factor of 77 ($K_D = 0.48$ nM and 37 nM respectively). Control experiments on separate DNA fragments^{18,23} reveal that neither a 5'-TGGCA-3' nor a 5'-TGGCCA-3' site is bound by polyamide **2** or **3** at concentrations ≤ 100 nM, indicating

Table 2 Pairing code for minor groove recognition

Pair	G·C	C·G	T·A	A·T
Im/Py	+	-	-	-
Py/Im	-	+	-	-
Hp/Py	-	+	+	-
Py/Hp	-	-	-	+

Favoured (+), disfavoured (-).

that the Hp/Py and Py/Hp ring pairings do not bind opposite G·C or C·G.

The discrimination between A·T and T·A is achieved when the two neighbouring base pairs are G·C and C·G (GTC compared with GAC). A general rule would require that the same discrimination be observed when adjacent A·T/T·A base pairs are present in the target sequence. Further sequence composition studies, which will be reported in due course, confirm A·T/T·A discrimination at sequence contexts which include TTT, TAA, TAT, TTA, ATT, GTT, GAT, and GTA steps (see Supplementary Information).

The specificity of **2** and **3** for sites that differ by a single A·T/T·A base pair results from small chemical changes. Replacing the Py/Py pair in **1** with a Py/Hp pairing as in **2**, a single substitution of C3-OH for C3-H, destabilizes interaction with 5'-TGGTCA-3' by 191-fold, a free-energy difference of 3.1 kcal mol⁻¹ (13.0 kJ mol⁻¹). Interaction of **2** with 5'-TGGACA-3' is destabilized only 6-fold relative to **1**, a free-energy difference of 1.1 kcal mol⁻¹ (4.6 kJ mol⁻¹). Similarly,

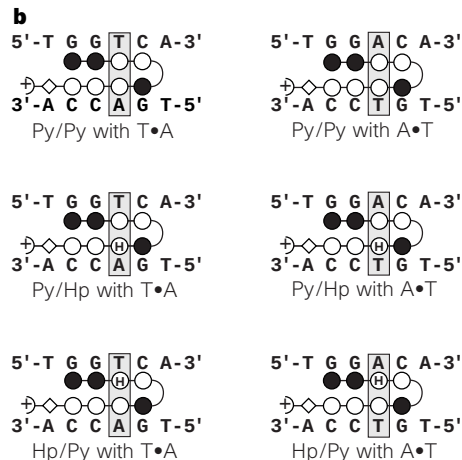
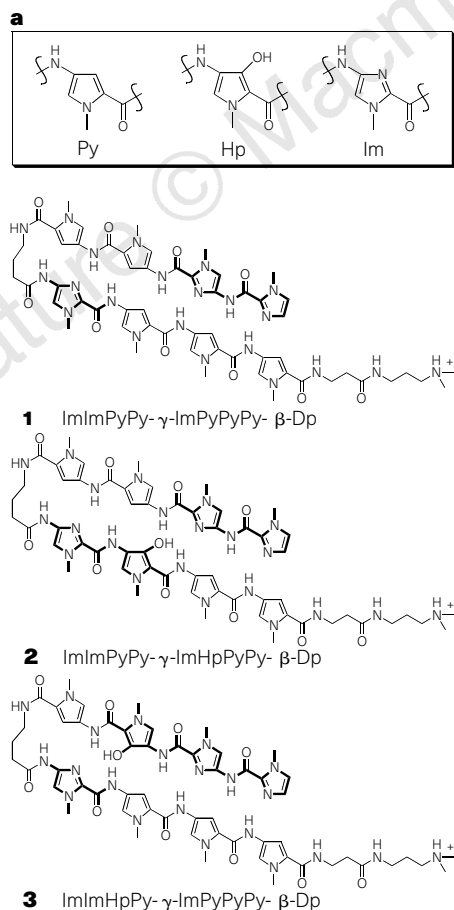


Figure 2 Eight-ring hairpin polyamides containing three aromatic amino acids (Py, Hp, Im). **a**, Structures of polyamides ImImPyPy-γ-ImPyPyPy-β-Dp (**1**), ImImPyPy-γ-ImHpPyPy-β-Dp (**2**), ImImHpPy-γ-ImPyPyPy-β-Dp (**3**). (Hp = 3-hydroxypyrrole, Im = imidazole, Py = pyrrole, β = β-alanine, γ = γ-aminobutyric acid, Dp = dimethylamino-propylamide.) Polyamides were synthesized by solid-phase methods using Boc-protected 3-methoxypyrrole, imidazole and pyrrole aromatic amino acids, cleaved from the support by aminolysis, deprotected with sodium thiophenoxide, (full synthetic detail will be described elsewhere) and purified by reversed-phase high-performance liquid chromatograph (HPLC)²⁸. The identity and purity of the polyamides were verified by ¹H NMR, analytical HPLC, and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS-monoisotopic): **1** 1223.6 (1223.6 calculated); **2** 1239.6 (1239.6 calculated); **3** 1239.6 (1239.6 calculated). **b**, Binding models for polyamide **1-3** in complex with 5'-TGGTCA-3' and 5'-TGGACA-3' (A·T and T·A in fourth position highlighted). Filled and unfilled circles represent imidazole and pyrrole rings respectively; circles containing an H represent 3-hydroxypyrrole, the curved line connecting the polyamide subunits represents γ-aminobutyric acid, the diamond represents β-alanine, and the + represents the positively charged dimethylaminopropylamide tail group.

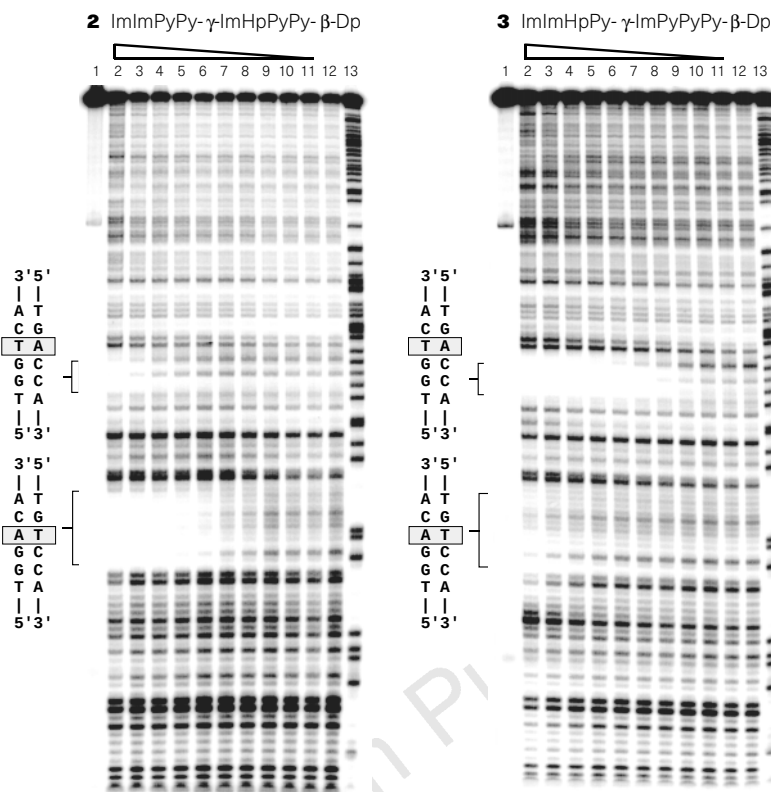


Figure 3 Quantitative DNase I footprint titration experiments. These experiments were made with polyamides **2** and **3** on the 3' 32 P-labelled 250-bp pJK6²⁹ EcoRI/PvuII restriction fragment. Lane 1, intact DNA; lanes 2–11 DNase I digestion products in the presence of 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 nM polyamide, respectively; lane 12, DNase I digestion products in the absence of polyamide; lane 13, adenine-specific chemical sequencing. All reactions were done in a total

volume of 400 μ l. A polyamide stock solution or H₂O was added to an assay buffer containing radiolabelled restriction fragment, with the final solution conditions of 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl₂, 5 mM CaCl₂, pH 7.0. Solutions were allowed to equilibrate for 4–12 h at 22°C before initiation of footprinting reactions. Footprinting reactions, separation of cleavage products and data analysis were carried out as described previously¹³.

replacing the Py/Py pair in **1** with Hp/Py as in **3** destabilizes interaction with 5'-TGGACA-3' by 252-fold, a free-energy difference of 3.2 kcal mol⁻¹. Interaction of **3** with 5'-TGGTCA-3' is destabilized only 6-fold relative to **1**, a free-energy difference of 1.0 kcal mol⁻¹. Determining the exact molecular basis for T:A recognition by Hp/Py must await high-resolution X-ray structure studies. For example, there is the possibility that the OH group of Hp is engaged in a hydrogen-bonding interaction with O2 of T.

Molecular recognition in nature occurs through an ensemble of stabilizing and destabilizing forces¹⁶. For example the DNA-binding transcription factor, TBP, recognizes 5'-TATA-3' sequences in the DNA minor groove via specific contacts and a large DNA-bend²⁴. For DNA-bending proteins such as TBP, practical sequence-specificity can be increased through the sequence-dependent energetics of DNA distortion²⁵. Homeodomain proteins recognize target sequences through a combination of specific interactions with both the major and minor grooves²⁶. An N-terminal arm recognizes 5'-TAAT-3' sequences in the minor groove such that a single substitution of T:A for A:T reduces binding at 5'-TTAT-3' by a factor of 7 (ref. 27). However, no single protein structure motif has been identified that provides a general amino acid–base pair code for the minor groove, although there has been remarkable progress with zinc-fingers in the major groove³⁰. Polyamides use a single molecular shape to provide for coded targeting of predetermined DNA sequences with affinity and specificity comparable to sequence-specific DNA binding proteins¹¹. Hydroxypyrrole–imidazole–pyrrole polyamides complete the minor-groove recognition code using three aromatic amino acids that combine to form four ring pairings (Im/Py, Py/Im, Hp/Py and Py/Hp) which complement the four Watson–Crick base pairs (Table 2). □

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A new perspective on the dynamical link between the stratosphere and troposphere

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Atmospheric processes of tropospheric origin can perturb the stratosphere, but direct feedback in the opposite direction is usually assumed to be negligible, despite the troposphere's sensitivity to changes in the release of wave activity into the stratosphere^{1–3}. Here, however, we present evidence that such a feedback exists and can be significant. We find that if the wintertime Arctic polar stratospheric vortex is distorted, either by waves propagating upward from the troposphere⁴ or by eastward-travelling stratospheric waves^{5,6}, then there is a concomitant redistribution of stratospheric potential vorticity which induces perturbations in key meteorological fields in the upper troposphere. The feedback is large despite the much greater mass of the troposphere: it can account for up to half of the geopotential height anomaly at the tropopause. Although the relative strength of the feedback is partly due to a cancellation⁷ between contributions to these anomalies from lower altitudes, our results imply that stratospheric dynamics and its feedback on the troposphere are more significant for climate modelling and data assimilation than was previously assumed.

To test for feedbacks from the stratosphere on the troposphere, we use a method known as piecewise potential–vorticity (PV) inversion. PV inversion more generally means the deduction of geopotential anomaly fields (and related fields such as the wind field) from fields of PV anomalies in the atmosphere and potential temperature anomalies at the Earth's surface. The inversion process requires solving a Poisson-like equation, which indicates that the non-local effects are qualitatively similar to the induction of an electric field by electric charge^{8,9}. It is a useful aid to understanding

atmospheric motions in circumstances where PV evolution is easily characterized. Piecewise PV inversion uses the superposition principle to calculate contributions to the geopotential height anomaly Z' attributable to PV anomalies in certain locations only¹⁰, analogous to calculating the electric fields that are due to only a subset of the total charge distribution.

The superposition principle is linear when the inversion is done on the basis of quasi-geostrophy (QG), and we accordingly use quasi-geostrophic PV^{11,12} (hereafter, simply PV) throughout this study. Quasi-geostrophy is based on the assumption that the magnitude of horizontal wind acceleration is smaller than the force due to the Earth's rotation (small Rossby number) and that the vertical stability is approximately uniform along a pressure–altitude. In QG, PV anomalies, q' , and geopotential height anomalies, Z' , are linearly related by

$$q' = \mathcal{L}(Z') \quad (1)$$

where $\mathcal{L}$ is a linear laplacian-type operator⁸. The linearity of equation (1) allows piecewise PV inversions to be performed unambiguously, as one can linearly superpose the contributions of individual PV anomaly 'pieces' to obtain the total Z' field.

Piecewise PV inversions have been applied in separate studies of tropospheric cyclogenesis^{10,13} and the stratospheric polar vortex¹¹. The latter research, which focused on how the stratospheric and mesospheric Z' fields are determined, included an exploratory analysis, using no tropospheric data, of the impact of two large stratospheric vortex deformations on the troposphere. Here we present an analysis and quantification of the influence of stratospheric PV anomalies relative to tropospheric PV anomalies in determining Z' in the upper troposphere. (The tropospheric PV anomalies include anomalies at the tropopause level: that is, those along the boundary between the troposphere and stratosphere.) Our study uses meteorological data for the winter of 1992/93 (analysed fields from the United Kingdom Meteorological Office, UKMO¹⁴) for both the troposphere and stratosphere, and diagnoses a broad range of cases of stratospheric polar vortex distortions, which are often associated with dramatic advective rearrangements of PV and ozone^{15,16}. The radiative effects of carbon dioxide, ozone and other greenhouse gases in the stratosphere, along with the breaking of planetary-scale Rossby waves, are responsible for the strong winds and horizontal PV gradients observed at the edge of the vortex^{17,18} which, when disturbed, can easily lead to strong advection. Stratospheric vortex distortions can be due to both the vertical propagation of planetary-scale Rossby waves from the

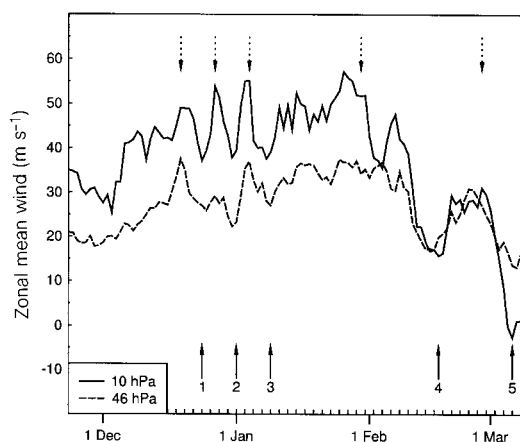


Figure 1 Time series of the zonal mean wind at 61.25° N for the winter of 1992/93. The solid line is for 10 hPa (~30 km) and the dashed line is for 46 hPa (~20 km). Up-arrows mark vortex disturbance events and down-arrows mark non-events. The events are numbered for reference in the text and other figures.

troposphere⁴ and the interaction of a stationary wave pattern with an eastward-travelling wave within the stratosphere^{5,6,19}.

We apply a piecewise inversion of q' over the Northern Hemisphere (north of 15°N with a lateral boundary condition of $Z' = 0$ at 15°N) to calculate Z' at the tropopause (taken as 215 hPa or about 11 km) induced by PV anomalies at individual atmospheric levels. (Throughout this paper, 'induced' is used in the electrostatic sense of an electric charge inducing an electric field; it is *not* meant to imply a causal relationship.) At the lower and upper horizontal boundaries of pressure-altitude, the vertical gradient of Z' is set proportional to the distribution of potential temperature anomalies (θ') (ref. 10). Surface θ' can be regarded as a PV anomaly concentrated at the surface²⁰. If the upper or lower boundaries are not part of the subset of pressure-altitude surfaces to be inverted, the vertical

gradient of Z' is set to zero at the boundaries¹⁰. In this way, we determine the tropopause Z' induced by the PV anomaly field at each pressure-altitude (including θ' at the boundaries).

QG is reasonable for pressure-altitudes below 10 hPa (about 30 km; refs. 12, 21, 22), which are the dominant levels in our calculations. In the region between the tropopause and the key stratospheric PV anomalies, our calculations indicate both small Rossby numbers and only weak latitudinal variations in vertical stability. Even if locally invalid, such as at the uppermost levels of our domain, QG PV inversions are accurate far-field solutions. To assess the impact of transient PV anomalies associated with polar vortex distortions, we calculate anomalies by subtracting out the wintertime-average fields (December to February), which include the QG reference state.

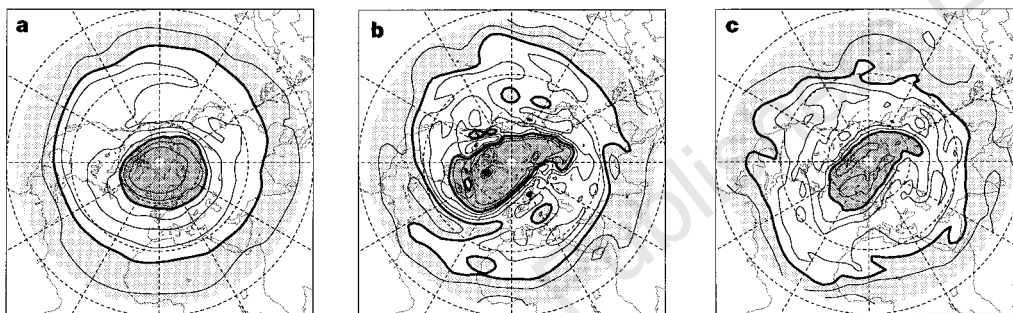


Figure 2 Quasi-geostrophic potential vorticity, q , for (a) 1992/93 wintertime mean at 10 hPa (~30 km) and for event 2 (1 January 1993) at (b) 10 hPa and 100 hPa (~16 km). Values range from 0 to $2 \times 10^{-4} \text{ s}^{-1}$. Bold dark lines correspond to $0.5 \times 10^{-4} \text{ s}^{-1}$ (the furthest south line) and $1.5 \times 10^{-4} \text{ s}^{-1}$ (the furthest north).

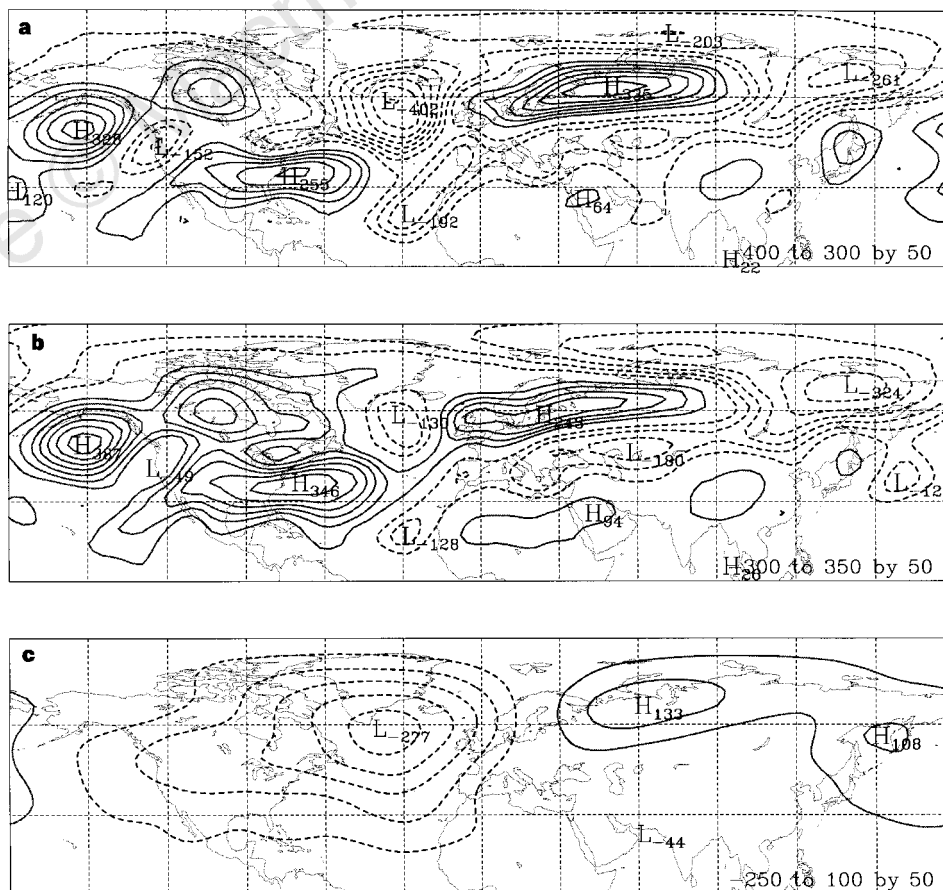


Figure 3 Geopotential height anomaly fields, Z' , on the Northern Hemisphere tropopause (215 hPa). a, Observed; b, Z' induced by the tropospheric PV anomalies, including boundary θ' ; and c, Z' induced by stratospheric PV

anomalies. 'Tropospheric' means pressure-altitudes of 1,000–215 hPa (~0–11 km) and 'stratospheric' means pressure-altitudes of 146–1 hPa (~14–50 km). The zero contour line has been omitted.

Polar vortex disturbances are associated with significant decreases in the zonal mean wind near the edge of the stratospheric polar vortex. Figure 1 shows a time series of this quantity at 61.25° N for the winter of 1992–93. If we define an ‘event’ as a drop in wind speed of at least 10 m s^{-1} at 10 hPa ($\sim 30 \text{ km}$) with a corresponding change at 46 hPa ($\sim 20 \text{ km}$), about five events occurred during this winter. ‘Non-events’ are defined as the times of maximum wind speeds between events (see Fig. 1). The PV distribution at 10 hPa for the wintertime average and for event 2 (January 1) are shown in Figs 2a and b, respectively. Figure 2c illustrates the vertical extent of the vortex distortion.

We first discuss the piecewise inversion for event 2. Figure 3 shows Z' at the tropopause during the event day. Figure 3b is the component of the tropopause Z' that is induced by PV anomalies located within the troposphere. It is clear that a large fraction of the Z' field is unaccounted for by tropospheric PV anomalies alone. The difference is due to the stratospheric influence shown in Fig. 3c. This component has magnitudes comparable to the Z' field induced by the tropospheric PV anomalies, indicating the large influence of stratospheric PV rearrangements on tropospheric dynamic fields.

To ascertain the location of the stratospheric PV anomalies that have the largest impact on tropospheric dynamical fields, we make use of a geopotential contribution function¹¹. This measures the fraction of Z' at the tropopause that is due to the integrated effect of

PV anomalies above a specified altitude. This is calculated by taking the covariance of the observed tropopause Z' with the tropopause Z' induced by PV anomalies above the specified altitude. It is then integrated horizontally and normalized to give a fractional contribution. Figure 4a shows the geopotential contribution function as a function of altitude for each of the five events. On average, 34% of the tropopause Z' is induced by stratospheric PV anomalies, with most of this contribution from the lower (~ 12 – 16 km) and middle (~ 17 – 24 km) stratosphere and nearly negligible amounts from the upper stratosphere (~ 25 – 50 km).

The correspondence between the strength of the zonal mean wind change (Fig. 1) and the stratospheric contribution to the tropopause Z' is evident from Fig. 4. The two largest events (4 and 5) show the strongest stratospheric contribution to tropopause Z' . In fact, during event 5 stratospheric PV anomalies account for more than half of the tropopause Z' . We also note that the contributions of θ' at the Earth's surface largely cancel the mid-tropospheric influence on tropopause Z' . This cancellation⁷ is evident in the contribution function, which exhibits a cancellation point at 7 km. In Fig. 4b, we plot the same contribution function for five non-events. For these cases, we find that, on average, about 15% of the tropopause Z' is induced by stratospheric PV anomalies. It is evident that the rearrangement of PV that occurs during vortex distortions induces strong geopotential height anomalies at the tropopause.

These results clearly indicate that stratospheric processes induce significant anomalies in dynamical fields at the tropopause. Although a coarsely resolved stratosphere is included in many current general circulation models and data assimilation systems used for tropospheric applications, our results suggest that a more realistic representation of the stratosphere may be required to enable such a model to properly simulate the troposphere. For upward-propagating linear Rossby waves, a suitable boundary condition at the tropopause may be sufficient to describe this feedback. But for internal stratospheric distortions and nonlinear breaking of vertically propagating Rossby waves, an atmospheric model must adequately represent the stratospheric processes in order to properly simulate the feedback on the troposphere. The feedback discussed here could significantly affect short-term and large-scale variations in the output from model simulations and data assimilations. □

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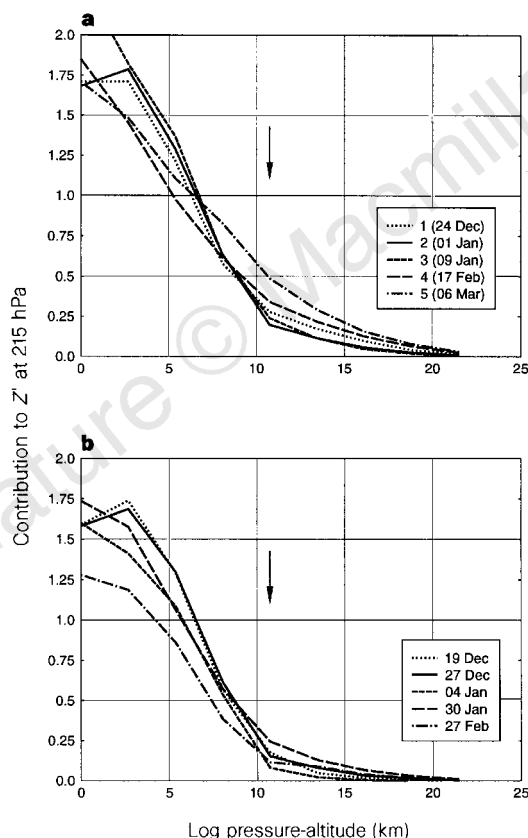


Figure 4 Geopotential contribution function as a function of log pressure-altitude for (a) events and (b) non-events. The geopotential contribution function is the normalized covariance of the observed Z' at the tropopause (215 hPa) with Z' induced by PV anomalies (q') located above a specified pressure-altitude. This is a measure of the fraction of the Northern Hemisphere tropopause Z' that is induced by PV anomalies above a specific pressure-altitude. Log pressure-altitude (z^*) is calculated from the UARS pressure levels by $z^* = -H \ln(\rho/\rho_0)$, where $H = 7 \text{ km}$ and $\rho_0 = 1,000 \text{ hPa}$. The legends specify the date and event number that each line represents. An up-arrow is located at the log pressure-altitude of the tropopause. If the surface-induced Z' was included at $z^* = 0$, the contribution function at $z^* = 0$ would be identically 1.0.

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Simulated future sea-level rise due to glacier melt based on regionally and seasonally resolved temperature changes

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Climate change is expected over the next century as a result of anthropogenic emissions of greenhouse gases and aerosols into the atmosphere, and global average sea level will consequently rise. Estimates¹ indicate that by 2100 sea level will be about 500 mm higher than today as a result of global warming, with thermal expansion of sea water accounting for over half of this rise. The melting of glaciers and ice sheets will contribute much of the remainder. We present an improved calculation of glacier melt, which uses the temperature patterns generated by a coupled atmosphere–ocean general circulation model^{2,3} as inputs to a seasonally and regionally differentiated glacier model^{4,5}. Under specified greenhouse-gas and sulphate-aerosol forcings, our model predicts that glacier melt equivalent to 132 mm of sea-level rise will occur over the period 1990–2100, with a further 76 mm from melting of the Greenland ice sheet. These figures fall within the range of previous estimates made using simpler models¹; the advantage of our approach is that we take into account the effects of regional and seasonal temperature

variations. Our inclusion of these effects increases the calculated glacier melt by 20%.

The atmosphere–ocean general circulation model (AOGCM) used in this work is the HADCM2 model of the Hadley Centre². Both the atmosphere and the ocean components are grid-point models with a spatial resolution of 3.75° of longitude by 2.5° of latitude. The Greenland and Antarctic ice sheets are represented by a permanent layer of snow. Like other AOGCMs, HADCM2 does not include any smaller ice caps or glaciers, which are assumed to have negligible climatic influence on the large scale.

Two simulations ('SUL' and 'GHG') were made of time-dependent climate change from 1860 to 2100 (refs 2, 3; Fig. 1) forced with estimates of the historical and future increase of greenhouse gases. SUL also included the effect of sulphate aerosols, which cool the climate system by reflecting solar radiation. The global average temperature rise from 1990 to 2100 was 2.7 K in SUL, 3.3 K in GHG. Previous results⁶ for the temperature rise if aerosol is included span the range 1.0–3.5 K, reflecting both the differences among the scenarios used for emissions for greenhouse gases and aerosols, and the uncertainty in the response of the climate system, particularly in the effect of aerosol.

The glacier model calculates the rate of change dV/dt of world glacier volume (expressed as the equivalent volume of liquid water) due to glacier melt during year i (time t_i) as

$$\frac{dV}{dt} = \sum_{j=1}^n A_j \left(\Delta T_s(\mathbf{x}_j, t_i) \frac{dB}{dT_s}(\mathbf{x}_j) + \Delta T_n(\mathbf{x}_j, t_i) \frac{dB}{dT_n}(\mathbf{x}_j) \right)$$

which is a sum over $n = 100$ glaciated regions (Fig. 2)^{4,5}. The j th region is located at $\mathbf{x}_j$ (longitude, latitude), and has glacier area A_j and time-independent mass balance sensitivities dB/dT_s and dB/dT_n (always negative) to changes ΔT_s and ΔT_n , with respect to the unperturbed climate, in the average temperature of the summer and non-summer months. ('Summer' is June–July–August in the Northern Hemisphere, December–January–February in the Southern Hemisphere.) The 'mass balance' B (dimensions of metres per year) of a glacier is the rate at which it is changing its total mass (water volume equivalent) divided by its surface area. A 'mass balance sensitivity' is simply the derivative of B with respect to a climate parameter that affects it.

The sensitivities depend on present local climatological precipitation, through a relationship derived from the results of an energy–balance model calibrated for a number of individual glaciers representing a variety of climate regimes⁷, taking into account their hypsometry (area–altitude distribution). Glaciers in regions of high precipitation have a higher mass-turnover and extend to

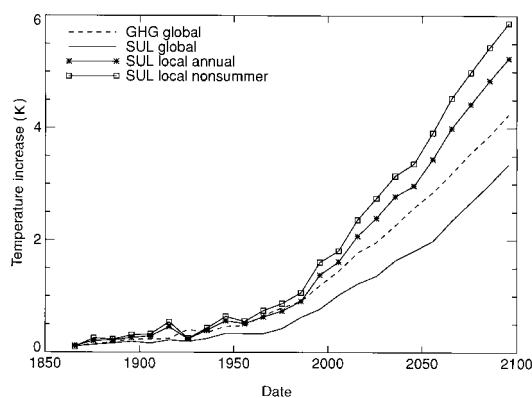


Figure 1 Simulated global-average temperature changes. From 1990 to 2100, carbon dioxide was increased in both GHG (dashed line) and SUL (solid lines) at 1% per year compounded, which is similar to the prescription of scenario IS92a of the Intergovernmental Panel on Climate Change (IPCC)¹⁵. SUL includes the additional effect of historical and future (IS92a) emissions of sulphate aerosols. All the temperatures shown are differences from the control integration, which has constant pre-industrial atmospheric conditions (nominally 1765). SUL and GHG began at 1860 with an instantaneous jump from the control. The simulated system adjusts to this with a characteristic timescale of about 6 years, so in a couple of decades it is very near to the state that would have resulted from a gradual increase of greenhouse gases before 1860. Starting in this way at 1860 leads to a loss of less than 10% in the time-integral of temperature 1860–1990. SUL and GHG provide a 'hindcast' of climate change since 1860, which Mitchell *et al.*³ compare with observations. They conclude that SUL gives a better match in both the global-average temperature change (about 0.5 K) and its geographical distribution in recent decades, whereas GHG overestimates the warming. However, both have discrepancies from the historical record. The local temperatures shown are averages over the glaciated regions only, where the annual average warming (asterisks) is larger than in the global average, and larger in non-summer months (squares) than in the annual average, as discussed in the text.

lower altitudes, where the temperature is above freezing for more of the year. Consequently they have a greater sensitivity to temperature change, and in some cases show substantial melting in non-summer months. The inclusion of the effects of seasonality and hypsometry on sensitivity is an important advantage of the model used here.

The glacier model excludes the effects of changes in precipitation, which could be important regionally (for example, the recent advance of glaciers in western Norway is related to increased precipitation), but temperature changes dominate on the large scale. Calculations with a mass-balance model⁸ indicate that a 20% decrease in precipitation would have the same effect as a temperature rise of 1 K. The temperature effect is likely to dominate; for instance, precipitation increases at only around 3% K⁻¹ in SUL and GHG north of 60° N.

The glacier model assumes the areas A_j are fixed, and hence that melt continues indefinitely at a constant rate for a given climate. In

fact, of course, the areas should decrease, as the glaciers contract and eventually disappear. This assumption is a weakness for which, in our opinion, there is currently no satisfactory solution. For the heavily glaciated regions and Greenland, from which most of the melt derives, it is probably acceptable for the next several decades. Further into the future our results represent an upper limit, giving an overestimate of perhaps 25% in the total melt by the end of the next century⁹. Uncertainties in the precipitation data and in the derivation of the sensitivities of the glacier model probably amount to 15% in the total.

To couple the models, time series of ΔT_S and ΔT_N are extracted for each x_j and used as input to the glacier model to obtain dV/dt . The rate of change of global average sea level dh/dt is computed using the total ocean surface area A_0 as

$$\frac{dh}{dt} = -\frac{1}{A_0} \frac{dV}{dt}$$

and the global average sea-level rise with respect to $t = 0$ is obtained by integrating dh/dt over time. Calculated by this method, the sea-level rise due to glacier melt over 1860–1990 is 19 mm from SUL and 33 mm from GHG, similar to the figure of 27 mm obtained by Zuo and Oerlemans⁵ using observed temperatures and the same glacier-melt model, and to Meier's¹⁰ estimate of 28 mm for 1900–1961.

The contribution to global average sea-level rise from glacier melt between 1990 and 2100 is 132 mm from SUL, 182 mm from GHG; the former is comparable with values of 120 mm and 160 mm obtained using simple climate models¹. The estimated present total volume of glaciers is equivalent to ~500 mm of sea-level rise¹, so by the end of the next century our predictions indicate that more than a quarter will have been lost. For comparison, sea-level rise from thermal expansion calculated by HADCM2 over the same period is 225 mm in SUL, 299 mm in GHG.

The largest contributions to total glacier melt derive from north-west America (43% of the total in SUL) and central Asia, whereas the intensively studied glaciers of the Alps contribute only about 1% of the total. All regions show less melting in SUL than in GHG, but the reduction is not uniform. Central Asia is significantly less important in SUL (23%) than in GHG (30%). This can be explained by the pattern of sulphate aerosol emissions, which in the scenario used is highly concentrated in the industrialized area of southeast Asia by the middle of the next century, reducing the temperature rise in this region relative to elsewhere.

Because the local seasonal temperature changes are approximately proportional to the global annual average $\Delta \bar{T}_A(t)$, there is a nearly linear relationship between dh/dt and $\Delta \bar{T}_A$. This gives 0.63 mm yr⁻¹ K⁻¹ for both SUL and GHG as our best estimate of the sensitivity to average temperature change of the glacier contribution to sea-level rise.

We repeat the calculation using the temperature change $\Delta T_A(x,t)$ averaged over the annual cycle at each location in place of both ΔT_S and ΔT_N . The result is 164 mm for SUL and 219 mm for GHG for 1990–2100, over 20% greater than from the first method. The reason for this has two parts. First, the annual average temperature rise in high northern latitudes, where most of the glaciers are, is greater than the summer temperature rise (Fig. 1), because there are additional feedbacks^{11,12} increasing the temperature rise in non-summer seasons. Second, most glaciers are more sensitive to summer than to non-summer warming, because a rise in temperature causes less increase in melt if the temperature remains low. These two facts together mean that the second calculation increases summer melt by more than it reduces non-summer melt. This effect is strongest for the Canadian and Siberian Arctic, less for Scandinavia and northwest America (Alaska, the Rockies and so on), whose comparatively maritime west-coast climates have a weaker seasonal contrast, and unimportant for the relatively low-latitude glaciers of central Asia (such as in the Himalayas).

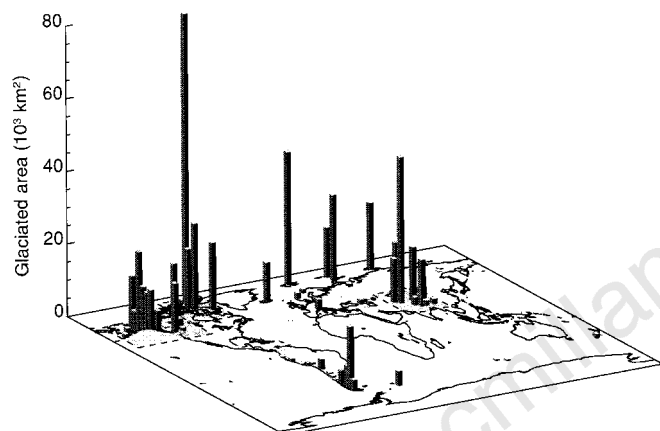


Figure 2 The locations of the 100 glaciated regions^{4,5}, excluding the ice sheets of Greenland and Antarctica. Each glaciated region is shown in its nearest AOGCM grid-box; some grid-boxes contain more than one. The three rectangular shaded areas are those we refer to as 'northwest America', the 'Canadian Arctic' and 'central Asia'. The precipitation characterizes the climate regime, which has a profound influence on the glacier mass-balance sensitivity. For instance, in the cold dry climate of the Canadian Arctic, the average magnitudes of summer and non-summer sensitivities are 0.15 and 0.01 mm yr⁻¹ K⁻¹ (expressed in terms of contribution to sea-level rise), whereas in the northwest American area, which is at lower latitude and affected by the nearby Pacific, there is much more non-summer melting, and the corresponding figures are 0.32 and 0.22. Iceland's mid-ocean location gives its ice caps a relatively mild and wet climate, with resulting sensitivities of 0.46 and 0.51.

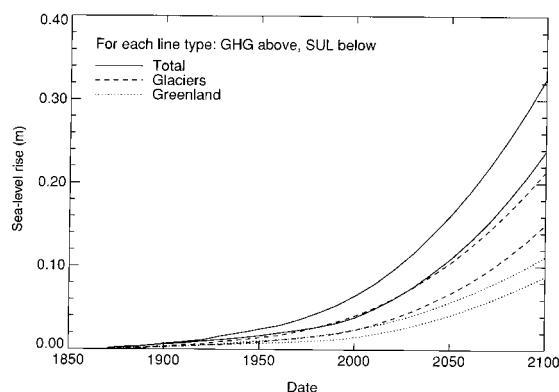


Figure 3 Contributions to global-average sea-level rise. Glaciers (dashed lines); Greenland (dotted); and their total (solid). Results are shown for both SUL and GHG; GHG gives the upper line in each case.

We perform the calculation a third time using ΔT_A at all locations and times of year. In this case, the result for 1990–2100 for SUL is 109 mm, GHG 149 mm, almost 20% less in each case than from the first method. This is because ΔT_A is less than the local temperature change for most glaciers, as the warming is generally larger over land than sea, and is greater in northern high latitudes.

Most of the water on land resides in the Greenland and Antarctic ice sheets, but the uncertainty about their state of mass balance is as large as the current observed rate of sea-level rise. However, it is relevant to consider the perturbations that might arise from anthropogenic climate change.

The Greenland ice sheet is similar to other high-latitude ice caps in that increased ablation will be the dominant effect of climate change on the mass balance. Therefore we extend the glacier model by defining four additional regions to cover Greenland, corresponding to different climate regimes¹³. They give a total sea-level rise of 76 mm for 1990–2100 in SUL, 93 mm in GHG. This is only about half the size of the glacier melt in each case (Fig. 3), despite the vastly greater area of the ice sheet, because its high latitude and altitude mean that its average surface temperature will remain low. Expressed as a temperature sensitivity, the contribution from Greenland to sea-level rise is $0.35 \text{ mm yr}^{-1} \text{ K}^{-1}$ in SUL and $0.30 \text{ mm yr}^{-1} \text{ K}^{-1}$ in GHG. Various other estimates¹ have been made of this sensitivity, mostly lying within a range of $0.30 \pm 0.15 \text{ mm yr}^{-1} \text{ K}^{-1}$, an uncertainty of 50%. As with mountain glaciers, we find that the total is increased by the use of ΔT_A , but reduced with ΔT_{ice} .

The Antarctic ice sheet experiences little ablation in either the current or future climates. Accumulation is mainly balanced by calving of icebergs, at a rate which is unlikely to change substantially over the next century, because it responds on the long timescale of ice-sheet dynamics. But precipitation over Antarctica apparently increases strongly with temperature, so climate change in Antarctica will make a negative contribution to sea level, as a consequence of the greater accumulation which will occur in a warmer climate. The sensitivity has been estimated¹ as $-0.30 \pm 0.15 \text{ mm yr}^{-1} \text{ K}^{-1}$, which would result in a sea-level fall of 79 mm over 1990–2100 with the temperature changes predicted by SUL, roughly cancelling the contribution from Greenland, as other studies have also suggested¹⁴. □

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Oceanic signals in observed motions of the Earth's pole of rotation

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Motion of the Earth's pole of rotation relative to its crust, commonly referred to as polar motion, can be excited by a variety of geophysical mechanisms¹. In particular, changes in atmospheric wind and mass fields have been linked to polar motion over a wide range of timescales, but substantial discrepancies remain between the atmospheric and geodetic observations^{1–4}. Here we present results from a nearly global ocean model which indicate that oceanic circulation and mass-field variability play important roles in the excitation of seasonal to fortnightly polar motion. The joint oceanic and atmospheric excitation provides a better agreement with the observed polar motion than atmospheric excitation alone. Geodetic measurements may therefore be used to provide a global consistency check on the quality of simulated large-scale oceanic fields.

Measurements collected through a variety of observational techniques have firmly established the existence of a wide spectrum of variability in the motion of the Earth's pole. Our planet as a whole conserves its angular momentum except for the known effects of external torques associated with lunisolar tides. Thus, the observed variability in polar motion, measured in the reference frame of the crust and mantle to which instruments are attached, results from angular-momentum exchanges between the mantle and the other main components of the Earth's dynamic system, including the atmosphere and the oceans. The changes in the angular momentum of the last two components may be associated with changes in either their mass fields, hence altering their moments of inertia, or their motion fields relative to the crust. Relevant for polar motion are the changes in the angular-momentum components about the two equatorial axes⁵—conventionally taken to point towards the Greenwich and 90° E meridians.

Taking advantage of the quality of available atmospheric operational analysis systems—which involve the combination of global atmospheric data sets and state-of-the-art numerical models to achieve an optimal estimation of the three-dimensional atmospheric state every 6 or 12 hours—a number of studies^{1–4} have established a clear relation between variable wind and mass fields and polar motion, for seasonal and shorter timescales. The correlation between atmospheric and geodetic time series, though significant, was however far from perfect, and most studies have suggested the potential importance of other sources of excitation, with the oceans being considered prime candidates.

Early attempts at determining the role of the ocean in the excitation of seasonal polar motion^{6,7}, based on models with simplified dynamics, very coarse resolution, no ocean-bottom relief, and limited geographical extent, were necessarily inconclusive because of the nature of the simplifying modelling assumptions. In the past decade, however, ocean modelling has progressed immensely, due to improvements in model formulation, external atmospheric forcing field, and computing power. Although an ocean operational analysis system equivalent to those available for the atmosphere has yet to be developed, several global ocean modelling efforts are currently underway. Recently, general circulation models based on full dynamics and thermodynamics have been reported to

yield non-negligible signals in oceanic angular momentum about the equatorial axes^{8,9}, when compared to the atmosphere. Similar results have also been obtained with a simple constant-density ocean model¹⁰. In that study, the addition of oceanic to atmospheric time series provided some improvement in the coherence with the observed polar motion, mainly at submonthly timescales, but the short extent of the period analysed (~ 1 year) prevented a more general link between changes in ocean flow and mass fields and polar motion from being established.

Here we report results from a much longer simulation covering the period January 1985–April 1996, obtained with a new model developed recently as a tool to study the general circulation of the ocean. Model formulation and numerical implementation are described in detail by Marshall *et al.*¹¹. For the present purpose the model was configured on a 1° grid with realistic topography over the latitude range $\pm 80^\circ$, and with 20 levels in the vertical with layer thickness ranging from 25 m at the surface to 500 m at depth¹². The model was started from the final state of a previous 3-year spin-up run, and driven by 12-hourly wind stress fields and daily fields of surface heat and freshwater fluxes from the National Centers for Environmental Prediction (NCEP) for the period 1985–96. In addition to the surface heat and freshwater fluxes, the model was relaxed towards monthly mean sea surface temperature (SST) fields (that served as the lower boundary condition in the NCEP analyses) and towards monthly Levitus *et al.*¹³ salt climatology. The relaxation coefficient was varying in space and time and represented an estimate of the sensitivity of the surface flux to the SST¹⁴. Ice was simulated by a thermodynamic ice model¹⁵ and biharmonic friction was applied to the momentum equations. Forcing by barometric pressure is not included, but for periods analysed here (longer than 10 days), the inverted barometer approximation should hold¹⁰. A detailed description of the model solution cannot be given here, but overall the model performs satisfactorily on the large scale when compared to observations^{12,16}.

To examine the effects of the ocean on polar motion excitation, we follow the formulation of Barnes *et al.*⁵ and calculate effective equatorial angular-momentum functions χ_1^O and χ_2^O (Fig. 1). Each χ includes a motion term due to changes in the velocity field (χ^V), and a mass term due to changes in bottom pressure (χ^P). The motion

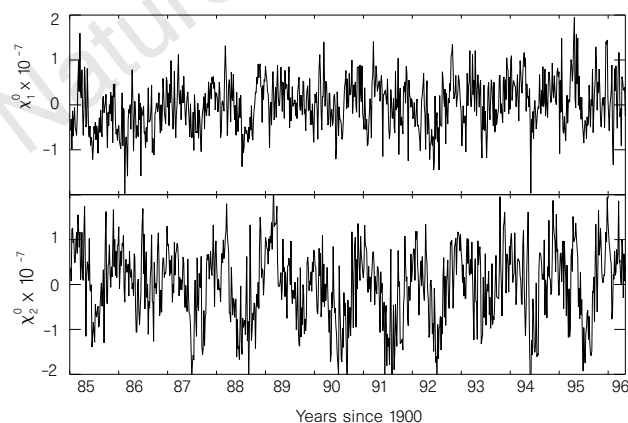


Figure 1 Five-day averaged values of χ_1^O and χ_2^O for the period January 85–April 96. Means have been removed. χ_1^O and χ_2^O are dimensionless and essentially proportional to the oceanic angular momentum about the two equatorial axes, conventionally taken to point towards the Greenwich and 90° E meridians, respectively⁵. The functions incorporate effects of the elasticity of the Earth and thus represent the effective excitation of polar motion by the oceans¹⁵. The time series show a rich variability over a wide range of timescales, and with no significant long term trends. The seasonal cycle is most conspicuous in χ_2^O , which tends to have slightly larger amplitudes than χ_1^O .

and mass terms were evaluated for the more than 11 years of model output, using 5-day averages of the velocity and bottom pressure fields, respectively. Bottom pressure was calculated based on the hydrostatic relation, using sea level and density estimated by the model, with the sea level corrected by a spatially constant, but time-variable, factor accounting for overall volume changes due to steric effects¹⁷. Both variable circulation and mass fields contribute to the oceanic excitation series, but there is more power in the pressure terms over most frequencies (Fig. 2), particularly in χ_2^P , a finding also characteristic of previous results from other models^{9,10}.

Corresponding time series for the effective atmospheric equatorial angular-momentum functions, χ_1^A and χ_2^A , based on the NCEP/NCAR (National Center for Atmospheric Research) reanalysis products, were obtained from the Sub-Bureau for Atmospheric Angular Momentum of the International Earth Rotation Service (IERS)¹⁸. Atmospheric χ^V included winds integrated up to the 10-mbar pressure level, and χ^P were based on the inverted barometer approximation¹⁸, consistent with our ocean modelling assumptions. Equivalent polar motion excitation functions, χ_1^G and χ_2^G , were computed from the EOP 97 C 04 pole positions reported by IERS, using the method of Wilson¹⁹. Five-day averaged time series were created from the original series to be consistent with the ocean products.

Comparison of the frequency spectra for χ^O and χ^A (Fig. 3) indicates that, although in general weaker than the atmosphere (the total power in χ_1^O and χ_2^O amounts to 70% and 32% of that in χ_1^A and χ_2^A , respectively), the oceans are a non-negligible source of polar motion over most frequency bands. Particularly important seem to be the seasonal signals in χ_1^O . Furthermore, the power density for the combined oceanic and atmospheric time series, χ^{O+A} , is consistently higher than χ^A alone across the entire spectrum (with the exception of the seasonal band for χ_2) and generally closer to the observed levels of polar motion excitation (χ^G). We note that for the seasonal band in χ_2 , atmospheric excitation is above what is observed. The addition of the oceanic excitation decreases the seasonal χ_2 power

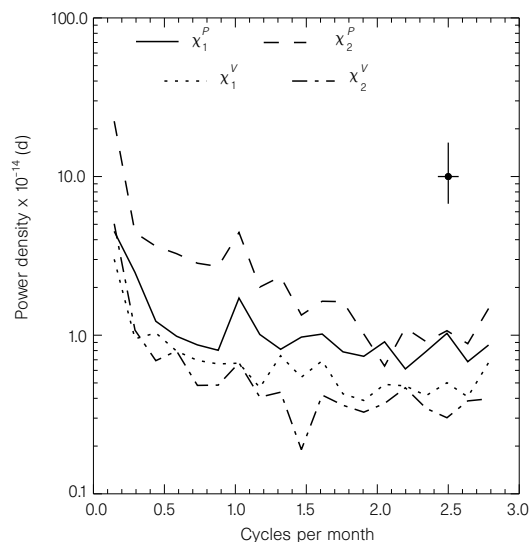


Figure 2 Power spectral density for oceanic χ_1^P (solid line), χ_1^V (dotted), χ_2^P (dashed) and χ_2^V (dotted-dashed) calculated by averaging respective periodograms over 20 adjacent frequencies. 95% confidence limits based on 40 degrees of freedom are shown on the upper right (vertical bar), together with the bandwidth (horizontal bar). The lowest frequency shown corresponds essentially to the seasonal band, including periods between 13.7 and 4.6 months. The differences between the various χ spectra reflect in part the particular choice of equatorial axes and the way oceanic variability projects on the spatially varying factors involved in each χ (refs. 1, 5). Regional contributions to the global integral represented by the χ^O values will be reported elsewhere.

density so that, over all bands, the power density in combined ocean–atmosphere excitation is below χ^G levels. Although the differences for each band are within the noise level, they could be made formally significant with further averaging in frequency. Therefore, the importance of other sources of excitation cannot be ruled out.

Besides contributing significant power to that available from atmospheric excitation alone, the inclusion of the oceanic series leads to significantly improved coherence with the observed polar motion excitation, for the frequency bands resolved (Fig. 3). Coherence amplitudes are consistently larger in the case of the joint atmosphere–ocean excitation, and phases are closer to zero, as expected for an improved agreement between geophysical excitation and observed polar motion. It is worth noting the case of the seasonal band for χ_1 , for which the addition of the oceans increases the coherence amplitude by 0.5, bringing it above the significance level and improving significantly the phase estimate. Overall, the correlation coefficients increase from 0.53 and 0.60 for the pairs (χ_1^A, χ_1^G) and (χ_2^A, χ_2^G) to 0.84 and 0.72 for the pairs (χ_1^{O+A}, χ_1^G) and (χ_2^{O+A}, χ_2^G) , respectively, and the root-mean-square difference between the paired series is reduced by $\sim 14\%$.

The present data (covering >11 years) are sufficient to separate the annual and Chandler periods (the latter is estimated¹ at 433 days) using harmonic analysis, but it is still too short a record to provide sufficient spectral resolution near those frequencies. Nevertheless, the amplitudes and phases of the harmonics at those periods (not shown) point to similar conclusions: the joint ocean–atmosphere excitation compares substantially better with the observed excitation at these periods than when only the atmosphere is considered. Determining with more certainty whether the excitation by the two geophysical fluids suffices to explain polar motion at these specific periods will require a longer record.

Even without considering the ocean's role, the presented coherence and correlation between χ^A , calculated from the NCEP/NCAR reanalysis, and χ^G , based on the recently created IERS data set,

represent an improvement over results from previous studies^{2,20,21}. The quality of the atmospheric and geodetic time series have, thus, considerably improved in recent years. With the inclusion of the oceanic signals, we are now approaching levels of agreement with χ^G much sought over the past decade, and similar to those obtained for the length of day²².

The ocean results reported here stem from modelling alone, without any estimation of the ocean circulation and mass fields by constraining the model to data. Nevertheless, the present significant improvements in the agreement with the observed polar motion, obtained when the simulated oceanic effects are added to the atmospheric excitation, attest to the high quality of the ocean model and its atmospheric surface forcing fields. The present study thus provides an unprecedented measure of consistency between three vastly different, but intimately coupled, dynamical systems, being modelled and observed at quite different levels of complexity. It also emphasizes the importance of the ocean in the Earth system, and the necessity to consider the effect of the ocean in Earth-related studies, including the planet's angular-momentum budget as well as fluctuations in the geoid.

Present simulations of the ocean excitation parameters are undoubtedly not free from error. Improved estimates of the oceanic χ functions are expected to come from efforts to incorporate available oceanic data in the estimation process, which are currently underway, as well as improvements in model physics and formulation, and forcing fields¹⁶. In particular, the inclusion of pressure forcing will probably be important¹⁰ as the scope of inquiry is extended to weekly and daily timescales. At these periods, accurate representation of the coupling forcing fields acting at the ocean–atmosphere interface will be crucial. $\square$

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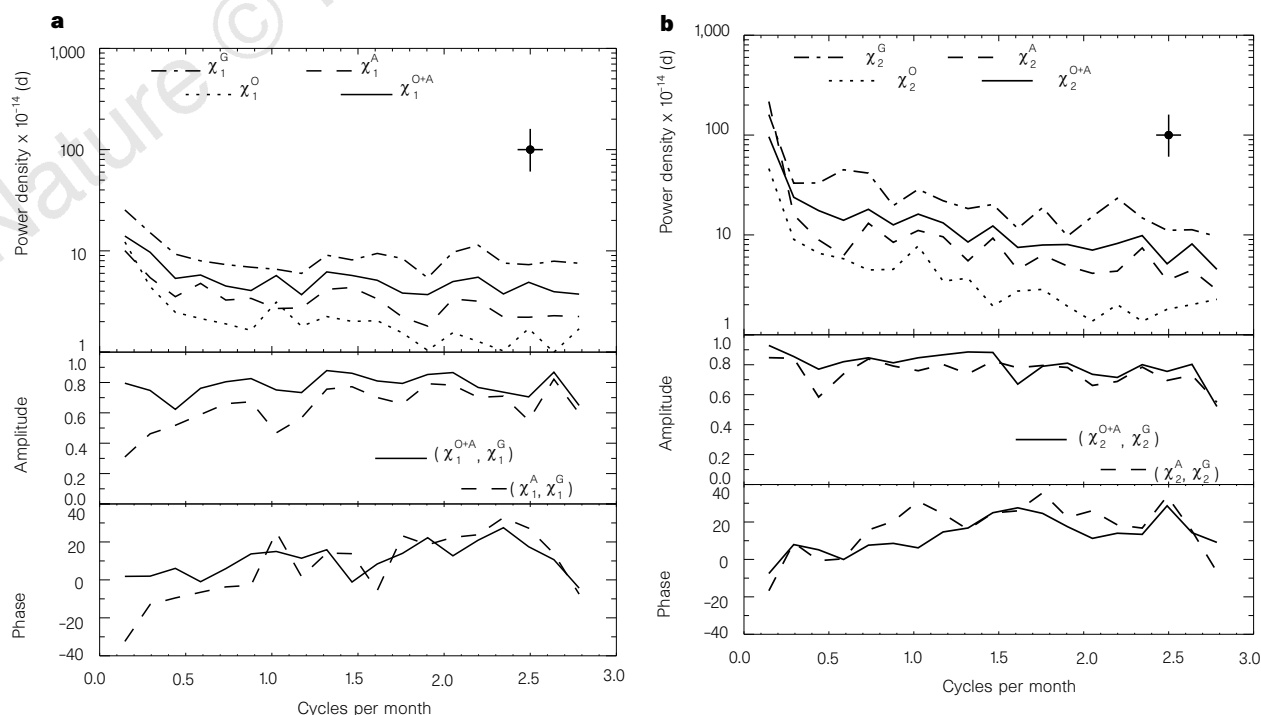


Figure 3 Assessment of oceanic effects on the excitation of polar motion. **a**, Power spectral density of χ_1^O , χ_1^A , χ_1^{O+A} , χ_1^G and coherence amplitude and phase in degrees for the pairs (χ_1^A, χ_1^G) and (χ_1^{O+A}, χ_1^G) . Similar curves for χ_2 series are shown in **b**. Power spectral density and coherence are calculated with band-averaging

over 20 frequencies. 95% confidence limits for the spectra are shown on the upper right (vertical bar), together with the bandwidth (horizontal bar). Coherence amplitudes >0.38 are significantly different from zero at the 95% confidence level.

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High hunting costs make African wild dogs vulnerable to kleptoparasitism by hyaenas

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The African wild dog *Lycaon pictus* is critically endangered, with only about 5,000 animals remaining in the wild¹. Across a range of habitats, there is a negative relationship between the densities of wild dogs and of the spotted hyaena *Crocuta crocuta*². It has been suggested that this is because hyaenas act as 'kleptoparasites' and steal food from dogs. We have now measured the daily energy expenditure of free-ranging dogs to model the impact of kleptoparasitism on energy balance. The daily energy expenditures of six dogs, measured by the doubly labelled water technique, averaged 15.3 megajoules per day. We estimated that the instantaneous cost of hunting was twenty-five times basal metabolic rate. As hunting is energetically costly, a small loss of food to kleptoparasites has a large impact on the amount of time that dogs must hunt to achieve energy balance. They normally hunt for around 3.5 hours per day but need to increase this to 12 hours if they lose 25% of their food. This would increase their sustained metabolic scope to a physiologically unfeasible twelve times the basal metabolic rate. This may explain why there are low populations of wild dogs in regions where the risk of kleptoparasitism is high.

African wild dogs are medium-sized carnivores (weighing ~25 kg) that live in packs of 4–20 adults and their dependent young. They feed predominantly on ungulates weighing 15–100 kg which they hunt and kill cooperatively. The members of a pack are normally nomadic within a large (~500 km²) home range. Wild dogs were formerly widespread south of the Sahara, but fewer than 5,000 individuals now survive¹. This serious decline in numbers has been blamed on several factors including habitat loss, persecution by humans³ and the transfer from domestic dogs of diseases such as rabies⁴. African wild dogs, however, live at low densities even in large areas of relatively undisturbed habitat, and their biomass is generally one to two orders of magnitude lower than that of competing spotted hyaenas². Across a range of habitats, there is a negative relationship between the density of dogs and hyaenas. Where the population density of hyaenas is high, and where visibility is good, for example on the Serengeti plains, these animals accumulate at the kills of dogs and reduce the dogs' rate of food intake^{5,6}. Hyaenas rarely take food from dogs in heavily wooded areas such as the Selous or Kruger Park, however^{7,8}. It is claimed that kleptoparasitism by hyaenas has been part responsible for the decline, or even demise, of wild-dog populations in open habitats⁹.

We used the doubly labelled water (DLW) technique¹⁰ to measure the daily energy expenditures (DEEs) of six fully grown dogs (three males and three females) from a pack living in the southwest of the Kruger National Park, Republic of South Africa. The pack consisted of 5 adults, 16 yearlings aged 16 months, and 27 pups aged 3–4 months. In the Kruger National Park, dogs generally hunt early in the morning and again towards dusk. At the time of the study, the dogs were away from the den and hunting for a total of 207 ± 15.1 min per day (mean ± 95% confidence interval (CI); $n = 59$ days). They spent the remainder of the day at rest. Daily energy expenditure, measured by DLW, averaged 15.3 MJ (Table 1). This is equivalent to a food intake of 3.5 kg of ungulate meat per day, assuming an energy content of 5.2 MJ per kg wet weight, a digestive efficiency of 93% and a 10% loss of energy in the urine. This figure agrees well with field determinations of rates of food intake, which range from 2.5 to 3.5 kg per dog^{7,8}. The high variability in DEEs among the six dogs probably reflects day-to-day variation in the involvement of individuals in the group hunt. As the study pack consisted of only 5 adults, together with 27 dependent pups and 16 yearlings who had just reached the age at which wild dogs hunt effectively, the pack might have been hunting rather more intensely than packs with a more favourable ratio of adults to young.

For comparison, allometric equations¹¹ predict a DEE of 6.0 MJ per day for a moderately active 25-kg domestic dog and 6.7 MJ per day for a highly active individual. The field metabolic rate of a 25-kg eutherian mammal has been predicted to be ~12.6 MJ per day¹². Measurements of food intake by working border collies¹³ give a value of 8.2 MJ per day for a 25-kg dog active for 6 hours per day. Alaskan sledge dogs weighing 25 kg used 47 MJ per day, as measured by both the DLW technique and the rate of food intake, on a 70-hour, 490-km sledge race across Arctic Canada¹⁴. By these comparisons, African wild dogs appear to be working extremely hard, despite the fact that they are active for only 3.5 hours per day. Moreover, the predicted basal metabolic rate (BMR) for a 25-kg dog

Table 1 Daily energy expenditures of six African wild dogs measured using the doubly labelled water technique

Dog	Sex	Mass (kg)	Plateau estimate (kJ per day)	Intercept estimate (kJ per day)
1	F	24	8,043	7,223
2	F	25	8,729	7,732
3	F	23	16,686	15,716
4	M	27	18,252	17,910
5	M	27	20,281	19,362
6	M	25	19,806	18,729

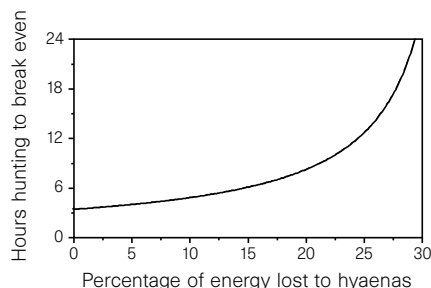


Figure 1 Model of the time for which wild dogs need to hunt each day as a function of the amount of food lost to kleptoparasites such as hyaenas.

is 3.4 MJ per day and thus the measured DEE represents a sustained metabolic scope of, on average, $5.2 \times \text{BMR}$. This is close to the suggested physiological limit on sustainable metabolic rates of around $6\text{--}7 \times \text{BMR}$ (refs 15–17).

We investigated the impact of kleptoparasitism using a model^{18,19} that predicts, from rates of energy expenditure when active and at rest and from rates of food intake, the length of time an animal must forage to obtain its daily energy requirements. The model takes the form $H_d = 24.E_r/(I + E_r - E_h)$, where H_d is the number of hours of foraging needed per day to achieve daily energy balance, I is the rate of capture of prey in kJ per hour, and E_r and E_h are the rates of energy expenditure when resting and when hunting, respectively, in kJ per hour. We have calculated E_r at 217.5 kJ per hour for a 25-kg dog, using an empirically derived formula for domestic dogs²⁰. This same formula predicts an E_r value of 2.74 MJ per day for an 8.5-kg wild dog; this value is close to a single, empirically measured value²¹ of 2.7 MJ per day. We assume that dogs metabolize at E_r for the 20.55 h per day that they spend at rest. Given a mean DEE of 15.3 MJ, this means that 25-kg dogs must be metabolizing at 3.14 MJ per hour (E_h) during the 3.45 hours that they are hunting each day. This is about $25 \times \text{BMR}$, which is close to the canid factorial scope. In the Kruger National Park, wild dogs rarely lose food to hyaenas. We assume, therefore, that they need to hunt for 3.45 hours to obtain their daily energy requirement of 15.3 MJ per day. The rate of food intake (I) averaged over this time is thus 4.43 MJ per hour.

We used these parameters to predict the numbers of hours that dogs would need to hunt if they were losing various percentages of their food to hyaenas (Fig. 1). As hunting is so energetically costly, a small loss of food to hyaenas has a relatively large effect on foraging times. For example, if dogs lost only 25% of their prey they would need to hunt for over 12 hours each day to achieve an energy balance. Dogs hunting for 3.5 hours each day are already working at close to their physiological limits, however. As hunting is so costly, a large extension of the time that they spend hunting would be physiologically untenable. This energetic limit on hunting times may explain why dogs are so vulnerable to even low levels of food theft. Attempts to conserve wild dogs will be most effective, therefore, in areas of thick vegetation, which restricts opportunities for kleptoparasites to detect kills, or where their main kleptoparasite, the spotted hyaena, is relatively rare. □

Methods

Field methods. Dogs were captured with minimum disturbance during the morning when they were resting in shade. They were anaesthetized with a 2.5 ml Telinject dart, fired from 10–20 metres, containing 2 mg fentanyl and 25 mg xylazine. Dashed dogs were ataxic within 3 min and recumbent within 7 min. During anaesthesia the dogs were quiescent, with mean heart and respiration rates of 33 and 9 per minute, respectively. The effects of fentanyl and xylazine can be completely reversed with an intravenous injection of yohimbine (0.125 mg per kg body weight) followed by 2.0 mg naloxone. After injection of the antidote, the dogs recovered within 45–90 s and then immediately ran off to rejoin the pack. At first capture the dogs were injected intravenously with 5 ml

DLW 60.6 APE (atom per cent excess) ^{18}O and 27.9 APE ^2H . Sequential 5 ml blood samples were taken at 20-min intervals over the next 3 h to establish the plateau of isotope enrichment. Five dogs were recaptured at 48 h and at 96 h and the other dog was recaptured at 96 h only; a 5-ml sample of blood was taken at each recapture.

Laboratory methods. Blood samples were distilled under vacuum using the pipette method²² and the ^{18}O and ^2H content of the derived water was measured by gas-source isotope-ratio mass spectrometry. The ^{18}O enrichment of the distilled water from each blood sample was determined by micro-equilibration of a weighed 10- μl water sample with 0.5 ml isotopically characterized CO_2 in an evacuated serological tube. After equilibration at 60 °C for 24 h, the CO_2 was removed and purified using a gas chromatograph on the front end of an Optima (Micromass) mass spectrometer. Three laboratory standards of known enrichment (relative to the international standards SMOW, SLAP and IAEA 302b) were run next to the experimental samples each day. ^2H enrichment of the injected water was determined by reducing 2 μl distilled sample water with optimally contaminated zinc (University of Bloomington, Indiana) in a sealed reaction vessel at 490 °C for 60 min. The evolved hydrogen gas was admitted directly into the mass spectrometer. Again, known laboratory standards were run daily. Enrichment of both isotopes was normalized to the SMOW–SLAP scale before conversion to parts per million using 0.0020052 and 0.00015595 as the best estimates of the absolute ratios of SMOW for ^{18}O and ^2H , respectively. For each dog, the blood enrichment of both isotopes decreased during the 3 h following injection. Twenty minutes after injection, estimates of body water derived from isotope dilution varied from 49% to 62%, suggesting that at this time the isotopes were not fully equilibrated. Between 1.5 and 3.0 h postinjection there was no significant change in isotope enrichment with time and the derived isotopic dilution spaces for oxygen were 65% to 71% of the body mass. This is close to the expected value for lean mammals²³. The isotope dilution space ratio was 1.0474 ± 0.011 (mean $\pm$ standard error) from the plateau estimates and 1.067 ± 0.014 from the intercept estimates. Both estimates are well within the range derived for other animals¹⁰. Neither differed significantly from the mean estimate of 1.037 for humans, but both were significantly greater than 1.0. The multiple-point intercept method and the two-sample plateau approach were used to derive the instantaneous elimination rates of oxygen (k_0) and hydrogen (k_d) for estimation of CO_2 production. We estimated CO_2 production using a two-pool model²⁴ which incorporates the mean dilution space ratio into the calculation. A recent validation study²⁵ of the DLW technique with domestic dogs confirmed that this equation compares best to simultaneous indirect calorimetry and food intake measurement. As CO_2 production is inversely related to the dilution space ratio, and as the observed dilution space ratio exceeded that assumed in equations derived for humans²⁶ or in equations frequently used in studies of animals²⁷, the use of any of these alternative equations would have resulted in higher estimates of CO_2 production. We converted CO_2 production to daily energy expenditure using a respiratory quotient value of 0.9 (22.8 kJ per litre of CO_2), which is appropriate for an obligate carnivore such as the wild dog.

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The representation of visual salience in monkey parietal cortex

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When natural scenes are viewed, a multitude of objects that are stable in their environments are brought in and out of view by eye movements. The posterior parietal cortex is crucial for the analysis of space, visual attention and movement¹. Neurons in one of its subdivisions, the lateral intraparietal area (LIP), have visual responses to stimuli appearing abruptly at particular retinal locations (their receptive fields)². We have tested the responses of LIP neurons to stimuli that entered their receptive field by saccades. Neurons had little or no response to stimuli brought into their receptive field by saccades, unless the stimuli were behaviourally significant. We established behavioural significance in two ways: either by making a stable stimulus task-relevant, or by taking advantage of the attentional attraction of an abruptly appearing stimulus. Our results show that under ordinary circumstances the entire visual world is only weakly represented in LIP. The visual representation in LIP is sparse, with only the most salient or behaviourally relevant objects being strongly represented.

Single neurons were isolated extracellularly in the LIP in two macaque monkeys. The visual responses and receptive field of each neuron was first assessed in a passive visual task in which visual stimuli were flashed during stationary fixation (Fig. 1a). Neurons were then tested with a stable-stimulus paradigm (Fig. 1b), in which a circular array of eight stimuli, which differed from each other in shape and colour, appeared at the beginning of an experiment and remained stably on the screen for its duration (usually more than 10 min or 100 trials). The array radius matched the eccentricity of the most active portion of the receptive field under study, so that

when the monkey made a saccade to the centre of the array, the neuron's receptive field was brought onto one of the array elements. In each trial a peripheral fixation point appeared (FP1 in Fig. 1b), situated such that no member of the array was in the receptive field when the monkey fixated it. This fixation point then stepped to the centre of the array (FP2 in Fig. 1b), and the monkey followed it with a saccade. The saccade brought the same symbol that had driven the cell so effectively into the receptive field, but the response of the cell was far less. To test whether this quiescence was due to the stability of the array stimulus we used a recent-onset variant of this task. In this variant only seven array symbols remained stably on the screen, and the eighth, the one that will enter the receptive field, was turned on anew in each trial while the monkey fixated the peripheral fixation point. After 500 to 2,000 ms, the monkey made a saccade that brought this recently appeared stimulus into the receptive field. The neuron now responded intensely (Fig. 1b, recent-onset stimulus). Of 31 neurons tested, 23 (74%) had significantly greater responses in the recent-onset condition than in the stable-stimulus condition (two-tailed *t*-test, $P < 0.05$). The median response after the saccade (in the 200-ms epoch beginning at the end of the saccade) across all 31 neurons was 29 Hz (range 1–139 Hz) in the recent-onset condition, and 17 Hz (range 0–76 Hz) in the stable-stimulus condition ($P < 0.001$ between conditions, Wilcoxon signed rank test). As can be seen in Fig. 1, the responses of some neurons were predictive, beginning earlier than would be expected from their visual latency alone, consistent with a previous report³. It is also clear from Fig. 1 that these responses did not depend on the execution of a subsequent saccade to the receptive field, as the monkey did not look at the stimulus in the receptive field in any of the trials shown.

These results demonstrate that the visual responses of LIP neurons are not simply due to the entry of a visual stimulus onto an appropriate retinal location. Instead, they are critically dependent on the abrupt onset of that stimulus, which renders it salient⁴. Recently appeared visual stimuli are represented in LIP even when they appear outside the receptive field and are brought onto it by a saccade, whereas stable stimuli evoke only weak or no responses.

Lights rarely flash in the world in which primates evolved. Most objects are stable in the environment and not intrinsically salient, but can become salient according to the needs of the animal. To determine whether stable stimuli are represented in LIP when they become behaviourally relevant we used a stable-target task in which all array stimuli were stable, but the monkey was required to make a saccade to just one of them on each trial. The monkey first fixated a peripheral fixation point, and then a cue appeared (outside the receptive field) that matched one member of the array (Fig. 2a). The monkey made a first saccade to the centre of the array, thereby bringing at least one array stimulus into the receptive field, and a second saccade to the cued array element, chosen pseudorandomly on each trial. When the cue informed the monkey that the second saccade would be to the stimulus entering the receptive field, the neuron discharged, starting around the first saccade and continuing until after the second saccade (Fig. 2Aa). In contrast, when the cue matched a stimulus outside the receptive field, the neuron did not respond, even though the same array stimulus entered the receptive field by means of the first saccade (Fig. 2Ab). Thus the neuron responded to the entry of a stable stimulus into its receptive field provided that the stimulus was behaviourally significant. In another version of the task the cue was presented after the stimulus had entered the receptive field (late-cue condition). Trials began when the monkey achieved central fixation. The cue was flashed during this fixation, and after a brief delay the monkey made a saccade to the cued array element. The neuron did not respond before cue presentation, when the stimulus had just entered its receptive field (Fig. 2B, first saccade). The neuron became active following cue presentation and continued until after the saccade, but only on trials in which the cue matched the stable stimulus in its receptive field

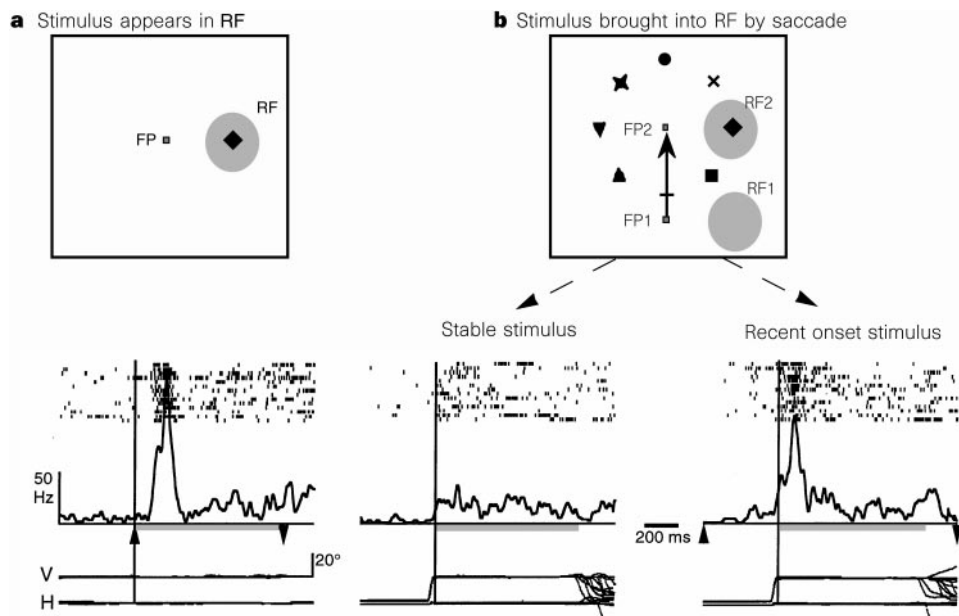


Figure 1 Effect of a recent onset on responses of one neuron. **a**, Neuron response when a diamond-shaped visual stimulus was flashed for 1 s at 15° right while the monkey maintained central fixation. The approximate location of the neuron's receptive field (RF) is indicated by the shaded area. **b**, Responses during the stable-array task. Top, illustration of the visual display at the time of the saccade from FP1 to FP2 (arrow). Eight symbols arranged in a circular array remained stably on the screen throughout collection of these data. Each subtended 2° and differed from the others in shape (as shown) and in colour (not shown). Fixation points were red squares, 2/3° on a side. The array was centred at the centre of gaze (FP2) and its radius matched each neuron's receptive-field eccentricity (in this case, 15°). During presaccadic fixation (at 20° down, FP1) the neuron's receptive field lay at position RF1, entirely outside the array. The saccade brought the receptive field to position RF2, overlapping a

stimulus physically identical with that used in **a**. The neuron had minimal responses in the stable-stimulus condition when all eight symbols remained stably on the screen. It responded strongly in the recent-onset condition when seven stimuli were stable but the diamond was turned on and off on each trial. Its firing rate in a 200-ms interval beginning at the end of the saccade was 20.8 ± 11.5 Hz (mean $\pm$ s.d.) in the stable-stimulus condition and 52.9 ± 23.6 Hz in the recent-onset condition. Arrowheads underneath each spike density trace indicate time of appearance and disappearance of the diamond-shaped stimulus. The grey line shows the time the stimulus was present in the neuron's receptive field. Raster lines, spike-density histogram and vertical (V) and horizontal (H) eye position are shown for each condition. Neural responses are aligned on the end of the saccade from FP1 to FP2 in **b** and on stimulus onset in **a**.

(Fig. 2Ba, b). Thus the response begins when the significance of the stimulus is established, whether that stimulus is just entering, or is already inside, the receptive field. The vast majority of neurons tested (78 of 82, 95% in the late-cue condition, and 29 of 29 in the early-cue condition) showed these response patterns.

We hypothesized that the responses in the stable-target tasks were related to the significance of the stimulus present in, or entering, the receptive field. However, LIP neurons are also known to have spatially selective presaccadic motor activity in the absence of recent visual stimulation⁵. To determine whether this presaccadic activity could fully explain the response in the stable-target task, we trained the monkey to make saccades into the receptive fields of the neurons in the absence of current or recent visual stimulation of the receptive field. Neurons were tested with a block of stable-target trials in which the cue always matched the stimulus in the receptive field. In the next trial block the saccade target was removed from the array, but all visual events were identical, and the monkeys were rewarded for making the same saccade as before, but now to an empty region of the array (no-target trials). Blocks of stable-target and no-target trials were interleaved. Neurons responded much more in stable-target than in no-target trials (Fig. 3a). Responses changed during the very first trial within a block and did not decrease systematically in subsequent trials, showing that the difference between the two conditions could not be an artefact of repeated trial presentation. Even though saccades in no-target trials usually had lower peak velocities and larger latencies than those in stable-target trials, there was no trial-by-trial correlation between firing rate and saccade velocity, amplitude or latency, neither within nor between experimental conditions. The decrement in neural

response between stable-target and no-target trials, therefore, was not related to differences in these saccade parameters (see Methods). We quantified the difference in neural response between the two conditions using the index $NT/(NT + ST)$, where NT denotes the response in no-target trials, and ST the response in stable-target trials (both measured in the 50-ms epoch ending at the onset of the saccade). We considered NT as a measure of the saccade-related or motor planning activity of the neuron, and any difference between NT and ST was thought to reflect processing of the visual target. The vast majority of indices fell below 0.5 (with $ST > NT$; median index, 0.39; mean, 0.40; range, 0.11–0.76; significantly different from 0.5, Student's *t*-test, $P < 0.001$; Fig. 3c, left). The median index corresponds to a reduction in response of almost 40% in the absence of the target. Consistent with previous findings, therefore, although some neurons have an independent saccade-related response, much of their presaccadic activity reflects the location of a selected visual stimulus rather than the planning of the saccade itself.

As a final control for our hypothesis that salient stimuli are represented in LIP regardless of the monkey's current motor behaviour, we used a version of the stable-target task in which the cues themselves flashed in the receptive field. In these trial blocks the cue matched, and dictated a saccade to a different array element on each trial, but saccades to the cue were never required. Nevertheless, the appearance of the cue elicited robust visual responses (Fig. 3b), which did not differ between trials in which the cue dictated a saccade to the target inside and outside the receptive field (TI and TO trials, respectively). The mean visual response across 34 neurons was 52 Hz in TI trials (median, 51 Hz; standard deviation, 43 Hz) and 47 Hz in TO trials (median, 47 Hz; standard deviation, 36 Hz;

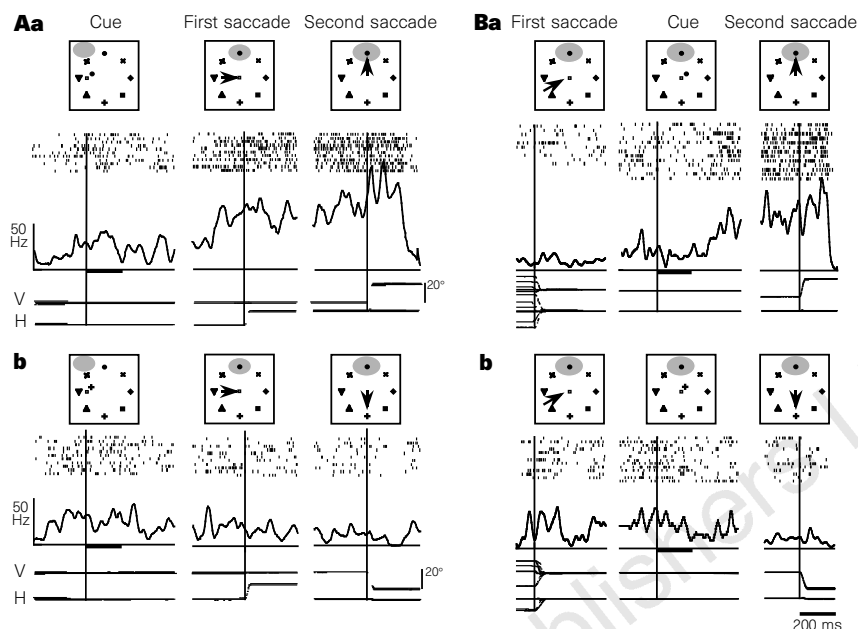
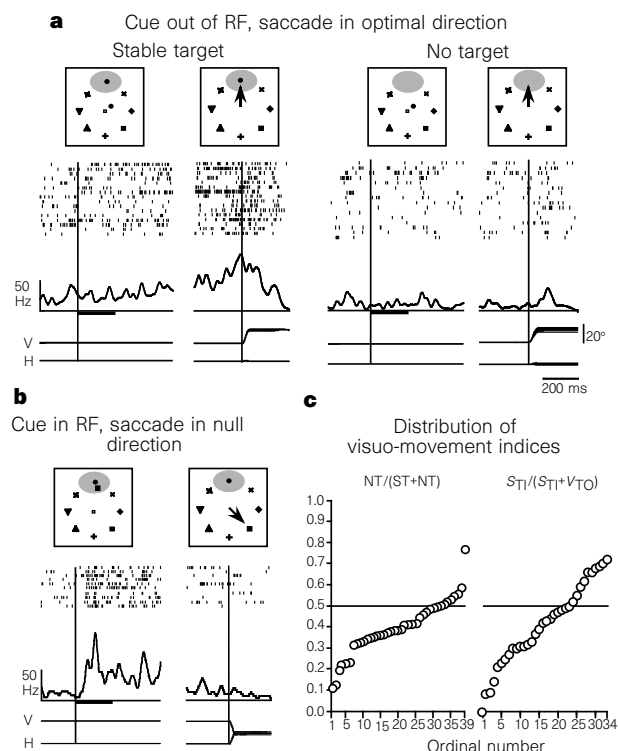


Figure 2 Responses of one neuron during the stable-target task. **A**, Early cue condition. While the monkey fixated a peripheral fixation point (15° left), where the receptive-field stimulus (the circle) was outside the neuron's receptive field, a cue was flashed for 200 ms 2° upward from this position (cue column, black bar). After a variable delay of 300–500 ms the fixation point jumped to the centre of the screen and the monkey followed it with a rightward saccade (first saccade column). After another 100–200 ms delay the fixation point disappeared and the monkey made a saccade to the array symbol matching the cue (second saccade). The matching symbol was chosen randomly among the eight array

elements, though only selected trial types are shown here. The neuron responded when the receptive-field stimulus (**a**), but not the opposite stimulus (**b**), was cued. Array radius, 18° . **B**, Late-cue condition. The monkey began each trial by fixating the centre of the screen, where its receptive field overlapped the receptive-field stimulus. The cue was presented 500–800 ms later for 200 ms at 2° above the fovea. After an additional delay of 300–500 ms, the fixation point disappeared and the monkey made a saccade to the cued array element. The neuron responded when the stimulus in the receptive field became the target for the saccade (**a**) but not otherwise (**b**).

Figure 3 a, No-target task. The neuron (same as that shown in Fig. 2) was tested with interleaved blocks of trials in which the saccade target was present (stable-target) or absent (no-target) from the array. The cue matching the stable target (circle) was presented on all trials at 2° up. Removal of the target greatly reduced the response of the neuron. Mean saccade amplitude was 14.5° in both blocks, with standard deviations of 0.5° in the stable-target condition and 1.5° in the no-target condition. Peak saccade velocity was significantly lower in no-target than stable-target trials (506 ± 63 and 599 ± 62 deg s^{-1} ; $P < 0.0001$, two-tailed t -test). **b**, Cue-in-receptive-field (RF) experiment. The neuron had a robust visual response to a cue that was flashed in the receptive field (at 10° up) but dictated a saccade away from the receptive field. Its response returned to baseline during the delay period. The task was identical to the late-cue condition shown in Fig. 2, except that all cues appeared in the receptive field. **c**, Indices $NT/(ST + NT)$ (left) and $S_{II}/(V_{TO} + S_{II})$ (right) are plotted in ascending order against ordinal number. See text for further details.



$P = 0.13$, Wilcoxon signed-rank test). Responses were directionally selective in the presaccadic period (50-ms epoch ending at the onset of the saccade). The mean presaccadic response was 37 Hz in TI trials (median, 33 Hz; standard deviation, 28 Hz) and 9 Hz in TO trials (median, 6 Hz; standard deviation, 11 Hz; $P < 0.0001$, Wilcoxon signed rank test).

For most neurons the visual responses to cues that dictated saccades opposite the receptive field (V_{TO}) surpassed the presaccadic responses for saccades to stable targets in the receptive field (S_{TI}). The distribution of indices $S_{TI}/(V_{TO} + S_{TI})$ for 34 neurons is shown in Fig. 3c. (Here V_{TO} is the average firing rate in the epoch 75–175 ms after cue onset, and S_{TI} is the average rate in the 50-ms epoch ending at saccade onset.) This index was smaller than 0.5 for most neurons, with $V_{TO} > S_{TI}$ (mean index, 0.41; median, 0.42; standard deviation, 0.19; $P = 0.01$ relative to 0.5, two-tailed Student's t -test). Because the presaccadic responses for saccades to stable targets were themselves greater than the presaccadic responses in the no-target task, these data suggest that most activity in the bulk of LIP neurons depends on the presence of a salient visual object, and does not simply reflect motor processing for saccades.

Taken together these experiments show that most LIP neurons have strong visual responses which are independent of saccade planning but depend critically on stimulus salience. Stimulus salience can either be intrinsic (produced by a recent abrupt onset) or dictated by the behavioural context. Evidence suggests that visual information that is of no immediate behavioural relevance is filtered out either in or before LIP. Thus neurons in primary visual cortex provide a more complete visual representation, discharging at high rates whenever saccades bring their receptive fields upon adequately orientated stable stimuli regardless of stimulus salience⁶. In contrast, visually responsive neurons in the frontal eye fields resemble LIP neurons in that they respond only to portions of complex, stable scenes that are targeted by the next saccade⁷.

Our data confirm and complement previous studies showing that visual responses coexist with saccade-related signals in individual LIP neurons. Recently, Snyder *et al.*⁸ studied a subset of LIP neurons that discharged during the delay period of a memory-guided saccade task. They showed that some of these neurons discharged less during the delay period of a memory-guided reach task, especially when the monkey simultaneously performed a saccade to a target in a different direction, and they hypothesized that LIP primarily encoded saccade preparation. The neurons in our sample with responses in the no-target task could participate in saccade preparation, and the projections from LIP to the intermediate layers of the superior colliculus and the frontal eye field^{9–11} are appropriate for that function. However, consistent with our findings, Snyder *et al.* also reported that the neurons responded to flashed visual stimuli independently of the monkey's current motor behaviour⁸. Our experiments show that these are not merely 'passive visual' responses but reflect the special salience of the recently appeared stimuli.

We suggest that the representation of visual salience in LIP may subserve a wide range of behaviours including, but not limited to, saccadic eye movements. Similar 'salience maps' have been postulated to guide a variety of behaviours including visual search with or without eye movements^{12–14}, the perception of unified objects¹⁵, and the phenomenon of positional constancy¹⁶. LIP could contribute to selective visual processing through its connection with visual areas, including V4, TE and TEO^{10,17}, which are thought to be important in pattern recognition and which have significant attentional modulation¹⁸. □

Methods

Experimental methods. Two male rhesus monkeys (*Macaca mulatta*) were prepared for physiological recording during sterile surgery under ketamine and isoflurane anaesthesia. All experimental protocols were approved by the NEI

Animal Care and Use Committee as complying with the guidelines established in the Public Health Service Guide for the Care and Use of Laboratory Animals. Behavioural control and data collection were done on personal computer using the REX system¹⁹. All physiological methods were as described⁵. Visual stimuli were projected upon a tangent screen by an Electrohome video projector driven by personal computer. Array members were 2° in diameter and varied slightly in luminance. Locations of recording sites were identified histologically in one monkey. In the second monkey, LIP neurons were recognized by their consistent visual, delay-period and saccade-related responses in a delayed-saccade task^{20,21}, and recording sites were localized to the intraparietal sulcus by magnetic resonance imaging. The distribution of neurons with visual, delay-period and presaccadic activity in our sample resembled that reported previously²¹.

Data analysis. Spike-density histograms were calculated by convolving the spike train, sampled at 1 kHz, with a gaussian of sigma 10 ms (ref. 22). Neural responses were measured as the average of this spike-density trace over the interval of interest, across all correct trials. To analyse the relation between saccade parameters (peak velocity, amplitude and latency) and the neural responses in the stable-target and no-target conditions, we fit the presaccadic responses of each neuron, for each condition (stable-target and no-target), with univariate least-squares linear regressions. Of 78 regressions (two each for 39 neurons), less than 5 were significant for each parameter. To further assess a possible relation between firing rate and saccade velocity at the population level, we computed a saccade velocity index $NT/(ST + NT)$ analogous to the response index described in the text. There was no correlation between the velocity and firing-rate indices across the 39 neurons.

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Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*

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The plant *Arabidopsis thaliana* (*Arabidopsis*) has become an important model species for the study of many aspects of plant biology¹. The relatively small size of the nuclear genome and the availability of extensive physical maps of the five chromosomes²⁻⁴ provide a feasible basis for initiating sequencing of the five chromosomes. The YAC (yeast artificial chromosome)-based physical map of chromosome 4 was used to construct a sequence-ready map of cosmid and BAC (bacterial artificial chromosome) clones covering a 1.9-megabase (Mb) contiguous region⁵, and the sequence of this region is reported here. Analysis of the sequence revealed an average gene density of one gene every 4.8 kilobases

(kb), and 54% of the predicted genes had significant similarity to known genes. Other interesting features were found, such as the sequence of a disease-resistance gene locus, the distribution of retroelements, the frequent occurrence of clustered gene families, and the sequence of several classes of genes not previously encountered in plants.

A region between markers COP9 and G3845 on the long arm of chromosome 4 from the ecotype Columbia was selected for sequencing. Figure 1 is a map of the position of 389 genes, predicted genes, retroelements and other features in the 1,874,503-base-pair (bp) sequenced region. The mean gene density of one gene every 4,806 bp is consistent with that determined in smaller regions sequenced on other chromosomes⁶ (*Arabidopsis thaliana* Database (AtDB) URL: <http://genome-www.stanford.edu/>). Assuming the coding regions of the *Arabidopsis* genome are 100 Mb, the total complement of *Arabidopsis* protein-coding genes can be calculated as ~21,000. Two hundred and seventeen (56%) of the predicted genes are similar to expressed sequence tags (ESTs) at the >95% sequence similarity level. Assuming there are ~12,000 unique *Arabidopsis* ESTs (<http://www.tigr.org/>), an independent assessment of the number of genes can be calculated as $389/217 \times 12,000 = 21,000$. This is consistent with the assessment based on gene density and predicted genome size.

Although the region sequenced here may be too small to detect chromosome-wide periodicity in features such as gene density, this feature varied between 7 genes in a 65-kb region (3400c to 3430w) to 12 genes in an adjacent 25-kb region (3435c to 3490c). Eighty-five per cent of predicted and experimentally determined genes contained multiple introns (from 1 to 29) which had no obvious distinguishing features apart from consensus donor and acceptor sites. Introns are 66.48% A+T compared to 55.96% A+T in exons, whereas intergenic regions are 67.77% A+T. There are no regions where experimentally determined genes overlap, but there are three instances of hypothetical genes encoded on the opposite strand to an experimentally determined gene. Putative promoter regions were often smaller than 200 bp, indicating that regulatory sequences may be found frequently in coding regions of other genes.

The degree of similarity between sequenced genes and those in the databases, assessed by their FASTA scores⁷, was used to classify genes. These classifications are shown in Table 1. Class 1 comprises 19 genes which had been sequenced previously; class 2 matches contain 73 genes that are highly similar (>1/3 FASTA self-score) to other genes, mostly from plants. Class 3 matches (242 predicted genes, 65%) comprise genes encoding proteins with a range of similarities to proteins of known function. The putative cellular

Table 1 Classes of similarities to genes

Class	FASTA score	Type of matching protein	Number	Predicted function
1	Identical	Same protein	19	18
2	>1/3 self score	Known protein	73	70
3a	<1/3 self score-150	Known protein	124	108
3b	150-80	Known protein	118	13
4a	>1/3 self score-150	Hypothetical protein	26	0
4b	150-80	Hypothetical protein	22	0
5		None but has EST match	0	0
6		None	7	
Total			389	209

Predicted genes were ordered into six classes, based on their degree of similarity to known or hypothetical proteins. The degree of similarity was assessed by comparing the self-FASTA score of the predicted protein sequence to the FASTA score resulting from a comparison of the predicted protein and its closest homologue. The value of the self-score relative to the comparative score compensates for differing lengths of the peptide sequences. The number of predicted genes in each class, and the number of predicted genes with assigned cellular roles, are also shown.

Table 2 Functional catalogue of plant genes

01 Metabolism	02 Energy	03 Cell growth/division	04 Transcription
01.01 Amino acid 01.02 Nitrogen and sulphur 01.03 Nucleotides 01.04 Phosphate 01.05 Sugars and polysaccharides 01.06 Lipid and sterol 01.07 Cofactors	02.01 Glycolysis 02.02 Gluconeogenesis 02.07 Pentose phosphate 2.10 TCA pathway 2.13 Respiration 2.16 Fermentation 2.20 E-transport 2.30 Photosynthesis	03.01 Cell growth 03.13 Meiosis 03.16 DNA synth/replication 03.19 Recombination/repair 0.322 Cell cycle 03.25 Cytokinesis 03.26 Growth regulators 03.99 Other	04.01 rRNA synthesis 04.10 tRNA synthesis 04.19 mRNA synthesis 04.1901 General TFs 0.41904 Specific TFs 04.1907 Chromatin modification 04.22 mRNA processing 04.31 RNA transport
05 Protein synthesis	06 Protein destination and storage	07 Transporters	08 Intracellular traffic
05.01 Ribosomal proteins 05.04 Translation factors 05.07 Translation control 05.10 tRNA synthases 05.99 Others	06.01 Folding and stability 06.04 Targetting 06.07 Modification 06.10 Complex assembly 06.13 Proteolysis 06.20 Storage proteins	07.01 Ions 07.07 Sugars 07.10 Amino acids 07.13 Lipids 07.16 Purine/pyrimidines 07.22 Transport ATPases 07.25 ABC-type 07.99 Others	08.01 Nuclear 08.02 Chloroplast 08.04 Mitochondrial 08.07 Vesicular 08.10 Peroxisomal 08.13 Vacuolar 08.16 Extracellular 08.19 Import 08.99 Others
09 Cell structure	10 Signal transduction	11 Disease/defence	20 Secondary metabolism
09.01 Cell wall 09.04 Cytoskeleton 09.07 ER/Golgi 09.10 Nucleus 09.13 Chromosomes 09.16 Mitochondria 09.19 Peroxisome 09.25 Vacuole 09.26 Chloroplast 09.99 Others	10.01 Receptors 10.04 Mediators 10.0404 Kinases 10.0407 Phosphatases 10.0410 G proteins 10.99 Others	11.01 Resistance genes 11.02 Defence-reglated 11.03 Cell death 11.04 Cell rescue 11.05 Stress responses 11.06 Detoxification 11.99 Others	20.1 Phenylpropanoids/ phenolics 20.2 Terpenoids 20.3 Alkaloids 20.4 Non-protein amino acids 20.5 Amines 20.6 Glucosinolates 20.99 Others
12 Unclear classification		13 Unclassified	Transposons
			14.01 LTR retroelements 14.02 Non-LTR retroelements 14.99 Other

Fifteen putative cellular roles for genes in plants are shown that are broadly based on the yeast functional catalogue⁹. New categories, adapted for plant-specific roles, have been made for secondary product metabolism and disease, defence and stress responses, and several new subcategories of genes involved in photosynthesis, chloroplast structure, storage processes and polysaccharide metabolism have been established. Categories between 14–19 have been reserved for gene categories in other organisms.

roles of most of the *Arabidopsis* genes encoding proteins with significant amino-acid similarity (FASTA > 150) over their entire length, or a high degree of conserved sequences over functional domains, were determined. Class 4 matches comprise 49 predicted genes (12%) similar to proteins of unknown function. No predicted genes were found in class 5 matches, representing predicted genes with cognate ESTs having no significant matches to any protein. Finally, class 6 matches were found for seven predicted genes that had no EST matches and no matches to sequenced genes. These genes are questionable because they are supported by neither experimental data nor similarity to other proteins. In total, the putative cellular roles of 54% of the predicted genes were established by sequence similarity to genes of known function in plants and other organisms. Plant genes accounted for 65% of the significant similarities, whereas cross-phylum matches with vertebrates (12%), bacteria and Archaea (10%) and yeasts (8%) accounted for most of the remainder. This distribution of matches emphasizes the extremely distant evolutionary relationship between the plant kingdom and other phyla.

Genes with predicted or known functions were classified into 15 putative cellular roles described in Table 2, which are based on the functional catalogues established for *Escherichia coli*⁸ and yeast⁹. The proportion of genes in each role category is shown in Fig. 2. Of the 206 genes analysed, the largest number were involved in primary and secondary metabolism (32%), reflecting the complex photo-autotrophic metabolism of plants. The 14% of genes involved in disease and defence responses may not be representative, because of the cluster of eight putative resistance genes at the *CHPR* gene cluster. The high proportion of genes involved in information processing (transcription 15%, and signal transduction 8%) are typical of complex multicellular organisms⁹. Table 3 shows the

predicted genes, their putative cellular role, the most closely related homologue, FASTA scores and cellular role category.

Five classes of repeat were encountered in the 1.87-Mb region: DNA sequence repeats in non-coding regions, retroelements, chloroplast DNA fragments, dispersed members of protein-coding families, and clustered repeats of related protein-coding genes. Together they comprise ~19% of the sequence. Several types of retroelement are found, in three configurations, in six places. First, two examples of a single copy of long terminal repeat (LTR) element, most similar to maize *hopscotch*¹⁰ (4045w and 4760c), were found distant from protein-coding genes, although 5045w is

Figure 1 This map shows the positions of genes, predicted genes and other features. A more detailed interactive representation is available via URL <http://www.mips.biochem.mpg.de/mips/athaliana/>. The annotated sequence has accession numbers Z97335–Z97344 inclusive. Predicted genes are numbered, starting at ATDL3000w on the proximal end of the contig, according to their position relative to the centromeric repeats which begin 2.3 Mb north of ATDL3000w. This allows for a minimum of 500 genes in this as-yet unsequenced region, with five digits to represent each predicted gene and any different versions. Genes are named according to standard conventions: AT, *Arabidopsis thaliana*; D, chromosome 4; L, long arm; w or c refers to the strand that encodes each protein. Genes are represented by rectangles pointing in the direction of transcription, starting at the ORF at the beginning of the predicted gene. The classes of matches of the predicted genes described in Table 1 are shown as different colours: class 1 genes are green, class 2 are turquoise, class 3a are pink, class 3b are red, class 4a and 4b are blue, and class 6 are dark blue. LTR repeats are blue pointed lines, non-genic sequence repeats are green pointed lines, tRNAs are red pointed lines, chloroplast DNA homologies are black pointed lines, and cognate cDNAs are turquoise pointed lines. The scale unit is 10 kb.

adjacent to a fragment of a retroelement, 4050w. Second, retroelements are found as adjacent pairs, such as 3275w, a TA1-3-like LTR retroelement¹¹, and 3275w, a TA11-1-like non-LTR retroelement¹¹, which are also distant from other genes. Finally, there are four examples of retroelement insertions in protein-coding genes. A TA11-1-like non-LTR retroelement (3835c) is inserted into the 5' end of the splicing factor homologue 3830c, and a remnant retroelement with homology over the finger protein (3840c) is adjacent to this element. A TA1-2-like non-LTR element, 3970c, is inserted close to the 3' end of a sesquiterpene cyclase gene (3975c). There are two insertions in *CHPR* putative disease-resistance genes described below. This pattern of dispersed single and pairs of retroelements in the low copy region of an *Arabidopsis* chromosome contrasts with the pattern of retroelements found in the larger genomes of plants such as maize, where retroelements of LTR- and non-LTR type form nested structures of multiple elements of different types that comprise 50% of the 2,400 Mb maize genome¹², in contrast to the total interspersed middle repetitive DNA that comprised 10% of the *Arabidopsis* genome¹¹. The expansion of retroelement numbers is proposed as the principal mechanism for increasing genome size in higher plants¹³.

Nearly 20% of the predicted protein-coding genes are members of gene families. The largest families include five predicted indole acetic acid glycosyl transferase genes, seven cytochrome P-450 proteins clustered in an 80-kb region, and a family of five closely related glutaredoxin genes clustered in 15 kb on the same DNA strand. Eight of the nine families comprise pairs of genes that are also adjacent on the same strand, and members of seven of these families were highly related. The frequent observation of close similarities among adjacent members of gene families on the same strand indicates that simple duplication and subsequent divergence may be a common mechanism for expanding gene families in *Arabidopsis*.

A 90-kb region encodes eight genes (4460c–4510c, 4525w) similar to the *Arabidopsis* ecotype Landsberg *erecta* (*Ler*) disease-resistance gene *RPP5* that confers resistance to the oomycete fungus *Peronospora parasitica* race Noco2 (ref. 14). The ecotype Columbia does not contain a functional *RPP5* gene; therefore the genes are named Columbia homologues of *Peronospora* resistance (*CHPR*). Two genes, 4490c and 4510c, contain frameshift mutations that would encode truncated, non-functional proteins, and 4500c lacks an in-frame ATG initiator codon. Two other *CHPR* genes contain retrotransposon insertions with terminal repeats in their coding regions. The Columbia -0 *RPP4* fungus-resistance gene maps to this locus¹⁴; therefore the two remaining genes, 4470c and 4505c (which has a cognate EST) could encode proteins conferring this resistance. A truncated kinase gene (4515c) is adjacent to intact putative resistance genes. It has a precise deletion of the first exon, compared to the intact *Ler* kinase orthologue (T.M. and J.D.G.J., unpublished), and fragments of the truncated kinase are found at the 3' end of all the *CHPR* genes, except for the truncated 4525w gene on the opposite strand. These fragments may be generated and amplified by recombination events within the putative resistance gene cluster. Putative resistance genes are found as single copies elsewhere in the sequenced region: 3600w is closely related to the *Solanum pimpinellifolium* Cf2.2 fungus-resistance gene, 3225c is related to the tobacco N TMV-resistance gene, and 3345c is closely related to the *Arabidopsis* RPS2 fungus-resistance gene. The precise disease resistance specificities of these genes, if any, are not known.

Five regions similar to the chloroplast genome were found: four were between 85–268 bp and were 70–80% similar, and a 2-kb tract, found between gene models 3690c and 3695c, has 80–94% similarity to chloroplast ribosomal protein L12 and tRNA^{Pro}. Finally, 53 mono-, di- and trinucleotide repeats between 20–50 bp were found, most commonly in introns.

A variety of genes were found that have either not yet been encountered in plants, or for which additional new examples will be of interest because they reveal conservation of additional cellular functions among sequenced organisms. Two genes have significant similarity to the cotton *celA1* (ref. 15) gene and may encode putative cellulose synthases. Two hydroxynitrile lyase homologues, 3595w and 4370c, were found that probably catalyse the first step in HCN production from cyanogenic glycosides. Many plants exhibit cyanogenesis as a deterrent to herbivores, but it was not known that *Arabidopsis* was among this group. Terpene cyclases catalyse the cyclization of allylic diphosphate substrates, and participate in a key regulatory step in the complex pathway of isoprenoid synthesis¹⁶. The adjacent genes 4390w and 4395w encode homologues of the monoterpene cyclase limonene cyclase, 3975c encodes a sesquiterpene cyclase homologue, and the adjacent genes 3715c and 3730c encode homologues of the triterpene cyclase lupeol cyclase. Gene 3390w encodes a protein with high homology to the carboxy-terminal half of HsORC1, the largest component of the DNA replication complex. An *Arabidopsis* complementary DNA encoding ORC2, another component of the putative initiator complex, has previously been characterized¹⁷. The presence of two of the possible six components of a complex that recognizes origins of DNA replication adds to the evidence for a replicon model of chromosome replication in *Arabidopsis*. 4725w encodes a protein that is 31% similar to of the *Drosophila* SPE1 protein, a DNA-mismatch repair enzyme related to the bacterial *mutS* and human *MSH2* mismatch repair superfamily¹⁸.

Among the genes encoding proteins involved in information processing, only 15% were found as cognate ESTs, reflecting the relatively low abundance of transcripts and possible differential expression of these genes. In this class, 4235c is notable as it encodes a 434-amino-acid protein 40% identical to yeast ADA2. ADA2 is required for the function of acidic activation domains of transcription factors in yeast, where it forms a complex with GCN5, a histone *N*-acetyltransferase¹⁹. The *Arabidopsis* HOOKLESS protein, involved in the late steps of ethylene signal transduction, is a member of the GCN5-related *N*-acetyltransferase (GNAT) superfamily²⁰, therefore it is possible that a protein complex analogous to the yeast ADA2–GCN5 transcription adaptor–chromatin modification complex may be found in *Arabidopsis*. Seven genes highly similar to genes involved in RNA processing were found. The

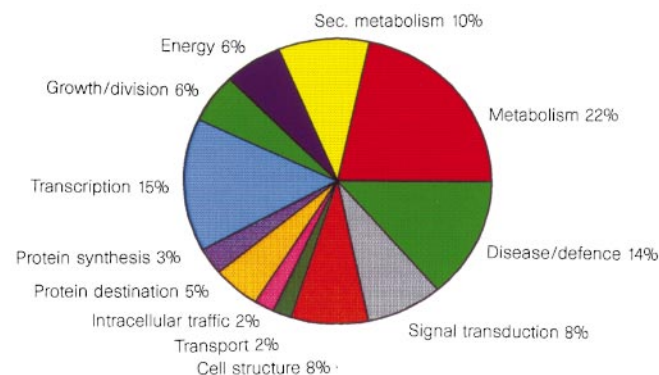


Figure 2 The pie chart shows the proportion of predicted genes with assigned cellular roles in each of the functional categories described in Table 2. A total of 209 genes were assigned to functional categories.

Table 3 A summary of genes with assigned cellular roles, based on significant similarities to genes of known function. The gene identifications are the same as in Fig. 1. The putative gene identity, closest homologue, their FASTA scores, and the functional category (described in Table 2), are shown. A more extensive list of homologues and scores is available through the PEDANT database at URL <http://www.mips.biochem.mpg.de/mips/athaliana/>.

high degree of similarity of the *Arabidopsis* homologues to the human RNA helicase 1 gene (4365c) and to the yeast SUV3 ATP-dependent RNA helicase (3435c) may indicate significant functional conservation of RNA processing mechanisms among eukaryotes. Two homologues of kinesins from *Caenorhabditis elegans* and *Xenopus*, 3115c and 3205c, show sequence conservation over the three functional domains, indicating that mechanisms of intracellular motility may be conserved between *C. elegans*, vertebrates and plants. Mutation of a putative Ca^{2+} -binding kinesin called ZWI-CHL caused morphological defects in trichomes²¹, demonstrating that this class of motor molecule contributes to cell shape in plants. Twelve per cent of the predicted genes found in the FCA region encode potential components of signal transduction pathways. For example, a homologue of a *C. elegans* calcium-sensor protein related to *Drosophila* frequenin, a calcium-binding protein that modulates neuronal efficiency²², is encoded by 4205c. Another *Arabidopsis* homologue of the yeast SNF-1 kinase, which regulates cellular sugar metabolism, is encoded by 3330c. 3280c encodes a protein kinase closely related to a developmentally regulated yeast kinase, *SPS1*, required for spore wall formation²³.

Four highly significant findings have been made in this pilot-scale sequencing project. First, there is a consistently high gene density over an extended contiguous region, with 389 predicted genes in 1.87 Mb. The relatively high gene density encountered in this region is also found in other sequenced regions. On the basis of this work and the 13 Mb of available sequence from four chromosomes, and the size of YAC contigs covering most of the low-copy regions of the five chromosomes, the total number of protein-coding genes is probably about 21,000. Second, the genome sequence has a high information content: 54% of the predicted genes can be assigned cellular roles on the basis of enzymatic, structural or other functions inferred by sequence similarity to proteins of known function. Nevertheless, the specific functions of most of these genes in plants requires further analysis. The remaining 46% of predicted genes, which either have no significant similarities to other genes or are similar to genes of unknown function, require extensive systematic experimentation to determine their cellular roles. Third, nearly 20% of the predicted genes are members of gene families that may have arisen by gene duplication and divergence. In other sequenced eukaryotes, such as *C. elegans* and yeast, the proportion is not as high. If the number of gene families in *Arabidopsis* is found to be ~15,000 after more comprehensive sequencing, *Arabidopsis* will have a similar-sized genome complement to the model metazoans, *Drosophila*²⁴ and *C. elegans*²⁵. This may represent a minimal number of genes required for the function of complex multicellular organisms with highly diverged mechanisms of development and environmental interactions²⁶. Finally, it is now clear that a straightforward shotgun-sequencing strategy can generate contiguous sequence from nearly all of the low-copy regions of the *Arabidopsis* genome. □

Methods

Contig assembly. The physical map of *Arabidopsis* chromosome 4 is represented by 4 YAC contigs covering 17 Mb (ref. 2). Sequencing was initiated at the FCA locus on the 13.5-Mb lower arm. The YAC map was used to assemble two cosmid contigs containing 1.2 Mb, using subcloning of YACs into cosmids in order to complete regions not represented in genomic cosmid libraries⁵. BAC clones were identified by hybridization to YAC clones and were assembled into the contigs to complete coverage²⁷.

Sequencing strategy. Sequence was determined from an overlapping contiguous set of 6 BAC clones, 15 Loris cosmids, 16 CC cosmids, 5 CAT cosmids, and 45 YAC subclones in the binary cosmid vector pCLD 04541. All libraries were prepared using DNA isolated from the Columbia ecotype. Clones were distributed in a network of 17 EU labs for sequencing, and the resulting sequence was assembled at the Martinsrieder Institut für Protein Sequenzen (MIPS). The quality controls used during sequence assembly involved comparison of 220,134 bp of overlap sequences, resequencing of selected

regions (90,566 bp), and resequencing of suspected low accuracy regions (8,684 bp), such as those harbouring potential frameshifts revealed by gene modelling. The total sequence produced was 2,094,637 bp, and the total non-redundant sequence was 1,874,503 bp. Shotgun sequencing of cosmid and BAC clones was the most common sequencing strategy.

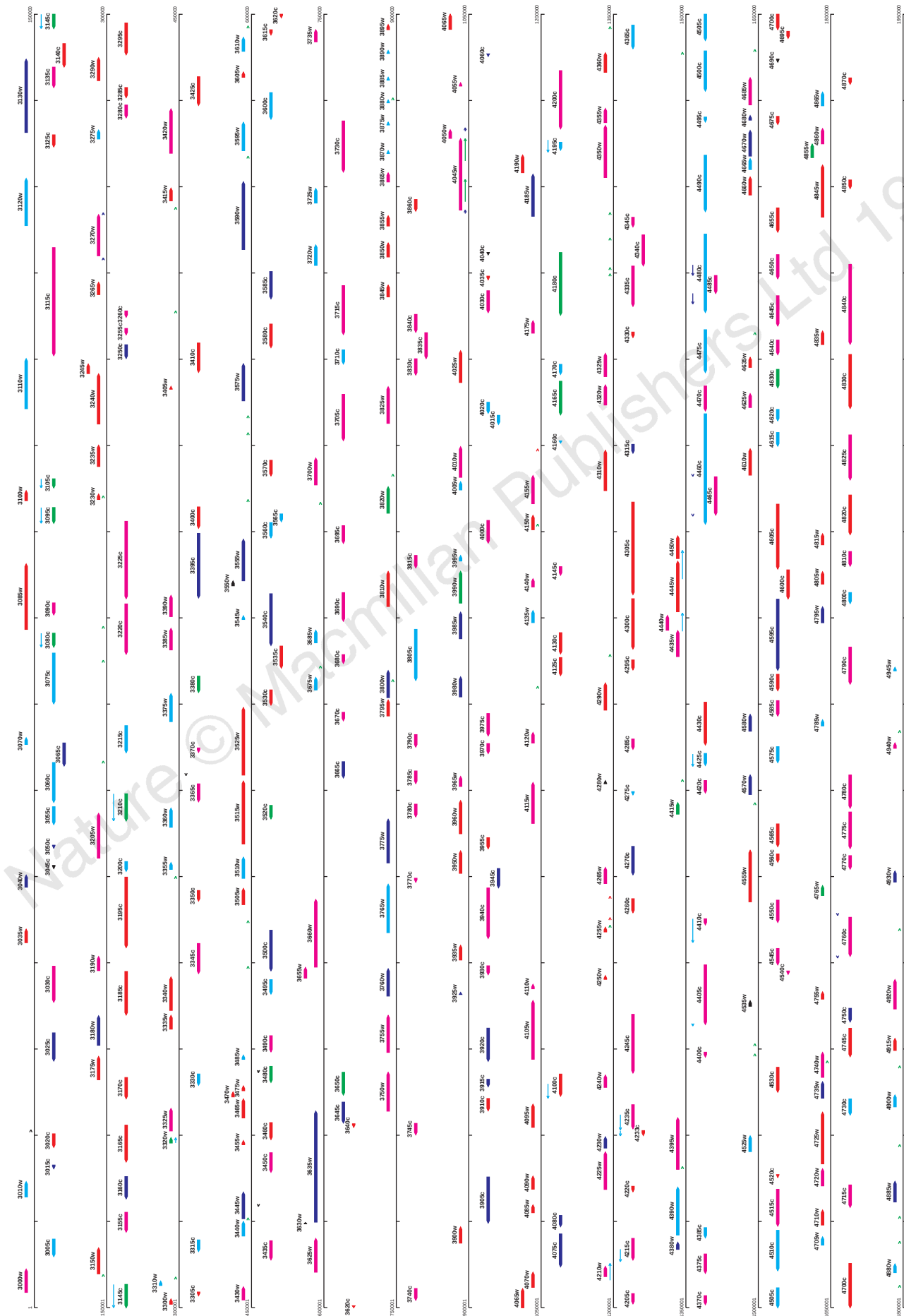
Sequence analysis. An initial BLASTX analysis²⁸ was used to compare all reading frames with all protein sequences and separately with the translations of *Arabidopsis* and other plant ESTs. A search for tRNAs and repeats was also made. Genefinder, Genmark and XGRAIL were modified using published *Arabidopsis* sequence and used for the identification of *Arabidopsis* genes. NetPlantGene was used for recognition of splice sites²⁹. Where possible, the predictions were checked for consistency with known protein sequences or cognate cDNA sequences, and the gene models adjusted accordingly. A total of 21,031 bp of cognate cDNA sequences were produced to aid modelling. The resulting protein sequences were extracted using FINDORFS for further analysis.

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Key:

-  class 1: proteins of known function
-  class 2: strong similarity to proteins of known function (more than one third of self-score)
-  class 3a: weak similarity to proteins of known function (FASTA score > 150)
-  class 3b: weak similarity to proteins of known function (FASTA score ≤ 150)
-  class 4a/b: weak similarity to proteins of unknown function (FASTA score > 150/≤ 150)
-  class 6: questionable ORF

-  non-genic sequence repeat
-  cognate cDNA
-  tRNA
-  LTR repeat
-  similarity to chloroplast genome

Table 3. A summary of genes with assigned cellular roles based on significant similarities to genes of known function

GENE	IDENTITY	FUN.CAT.	HOMOLOG	FASTA	SELF FASTA
ATD13005C	SERINE HYDROXYMETHYL TRANSFERASE -LIKE	1.01	A42906 PEA SERINE HYDROXYMETHYL TRANSFERASE	1355	2339
ATD13010W	ADENOSYLHOMOCYSTEINASE	1.01	SAHH_MEDSA ADENOSYLHOMOCYSTEINASE	2290	2411
ATD13480C	CYSTINE SYNTHASE	1.01	S65533 ARATH. CYSTINE SYNTHASE	1473	1473
ATD13495C	HIS7 -LIKE	1.01	HIS7_ARATH. IMPIDAZOLEGLYCEROL-PHOSPHATE DEHYDRATASE	1001	1183
ATD14945W	ACETYLTHIOESTERININE DEACETYLASE -LIKE	1.01	ARGE_DICDI DICTYOSTELIUM ACETYLORNITHINASE	212	602
ATD13380C	ATP SULPHURYLASE	1.02	S68201 ARATH. ATP SULPHURYLASE	2292	2299
ATD13110W	DNA CYTOSINE 3 ME TRANSFERASE -LIKE	1.03	S59604 ARATH. DNA CYTOSINE 3 METHYL TRANSFERASE	5516	7550
ATD14715C	PUR U -LIKE	1.03	S74352 SYNCHOCYSTIS PUR U	422	1497
ATD13080C	BETA 1,3 GLUCANASE	1.05	S31906 ARATH. BETA 1,3 GLUCANASE	2401	2401
ATD13105C	XYLOGLUCAN ENDOTRANSGLYCOSYLASE	1.05	S71226 ARATH. XTR7 XYLOGLUCAN ENDOTRANSGLYCOSYLASE	1515	1520
ATD13650C	BETA AMYLASE	1.05	S36094 ARATH. BETA AMYLASE	2533	2549
ATD13675W	UTP-GLUCOSE GLUCOSYL TRANSFERASE -LIKE	1.05	S49150 CASSAVA UTP-GLUCOSE GLUCOSYL TRANSFERASE	1005	2241
ATD13680C	UTP-GLUCOSE GLUCOSYL TRANSFERASE -LIKE	1.05	S41950 CASSAVA UTP-GLUCOSE GLUCOSYL TRANSFERASE	389	1553
ATD13685W	UTP-GLUCOSE GLUCOSYL TRANSFERASE -LIKE	1.05	S49150 CASSAVA UTP-GLUCOSE GLUCOSYL TRANSFERASE	1116	2390
ATD13690C	CELLULOSE SYNTHASE -LIKE	1.05	GHU58283_1 COTTON CELA1 CELLULOSE SYNTHASE	307	3774
ATD13705C	CELLULOSE SYNTHASE -LIKE	1.05	GHU58283_1 COTTON CELA1 CELLULOSE SYNTHASE	342	1402
ATD14030C	PECTIN METHYLESTERASE -LIKE	1.05	PC4168 ARATH. PECTIN METHYLESTERASE	980	3333
ATD14105W	GALACTOKINASE -LIKE	1.05	S27988 HAEMOPHILUS GALACTOKINASE	330	5102
ATD14170C	BETA 1,3 GLUCANASE -LIKE	1.05	S65077 PARA RUBBER BETA 1,3 GLUCANASE	910	1784
ATD14330W	GLUCOSYL TRANSFERASE -LIKE	1.05	CF11010_8 C.ELEGANS GLYCOCENIN GLUCOSYL TRANSFERASE	288	4627
ATD14325W	GLYCOCENIN -LIKE	1.05	Z72514_A_C.ELEGANS T10B10.8	289	2341
ATD14575C	BETA AMYLASE -LIKE	1.05	D50866 SOYBEAN BETA AMYLASE (EC 3.2.1.2)	1178	2492
ATD14625W	BETA 1,3 GLUCANASE -LIKE	1.05	A26668 ARATH. BETA 1,3 GLUCANASE	554	2998
ATD14720W	INOSITOL 2 DEHYDROGENASE -LIKE	1.05	I10511 B.SUBTILIS INOSITOL 2 DEHASE	175	1818
ATD14920W	TREHALOSE 6P SYNTHASE SUBUNIT -LIKE	1.05	U07184 ASPERGILLUS NIGER TREHALOSE 6P SYNTHASE	966	4392
ATD13255C	CARNITINE RACEMASE -LIKE	1.06	I41014 E.COLI CARNITINE RACEMASE	164	1139
ATD13260C	CARNITINE RACEMASE -LIKE	1.06	I41014 E.COLI CARNITINE RACEMASE	181	1124
ATD13255W	ACYLAMINOACYLPEPTIDASE -LIKE	1.06	I10132 PORCINE ACYLAMINOACYL PEPTIDASE	288	2104
ATD13610W	CTP-PHOSPHOCHOLINE CYTIDYLYLTRANS -LIKE	1.06	U19541 ARATH. CTP PHOSPHOCHOLINE CYT TRANSFERASE	479	1394
ATD13765W	FATTY ACID HYDROPEROXIDE LYASE -LIKE	1.06	U67996 ARATH. ALLENE OXIDE SYNTHASE	1571	3889
ATD14010W	2-HYDROXYHEPTA-2,7 DIONATE ISOMERASE -LIKE	1.06	FA4506 2-HYDROXYHEPTA-2,7 DIONATE ISOMERASE	362	1782
ATD14015C	EPOXIDE HYDROLASE -LIKE	1.06	D16268 ARATH. EPOXIDE HYDROLASE	704	1924
ATD14020C	EPOXIDE HYDROLASE -LIKE	1.06	U02495 SCLAMUM TUBEROSUM EPOXIDE HYDROLASE	479	2810
ATD14145C	3-OH BUTYRYL COA DEHYDRATASE -LIKE	1.06	CRT_CLOAB CLOSTRIDIUM CROTANASE	362	1109
ATD14375C	PHOSPHATIDYL SERINE DECARBOXYLASE -LIKE	1.06	A38732 HAMSTER PHOSPHATIDYL SERINE DECASE	260	2218
ATD14405C	ACTYL COA OXIDASE -LIKE	1.06	J2066 HUMAN ACTYL COA OXIDASE PEROXISOMAL	1462	4508
ATD14425C	ENOLYL COA HYDRATASE -LIKE	1.06	I37595 HUMAN ENOLYL COA HYDRATASE	530	2016
ATD14435W	TRIACYL GLYCEROL LIPASE -LIKE	1.06	IQ1390 RHIZOPUS TRIACYLGLYCEROL LIPASE	157	2935
ATD14630C	FARNESYL DIPHOSPHOSPHATE SYNTHASE -LIKE	1.06	S71182 ARATH. FARNESYL DIPHOSPHOSPHATE SYNTHASE	1694	1804
ATD14770C	PALMITOYL-PROTEIN THIOESTERASE -LIKE	1.06	U05315 D.C.ELEGANS PALMITOYL COA THIOESTERASE	214	1471
ATD14775C	PALMITOYL-PROTEIN THIOESTERASE -LIKE	1.06	U05315 D.C.ELEGANS PALMITOYL COA THIOESTERASE	469	2810
ATD13145C	PHYTOENE DESATURASE	1.07	CRT1_ARATH. PHYTOENE DEHYDROGENASE	1451	2488
ATD14665W	LACTATE DEHYDROGENASE -LIKE	2.01	D13817 RICE LACTATE DEHYDROGENASE	1153	1615
ATD13355W	GERMIN PRECURSOR OKALATE OXIDASE -LIKE	2.13	S71254 ARABIDOPSIS GERMIN TYPE 2	715	1009
ATD13465W	FERREDOXIN [2Fe-2S] -LIKE	2.2	FENM11 ARABIDOPSIS FERREDOXIN	245	570
ATD13870W	GLUTAREDOXIN -LIKE	2.2	S54825 CASTOR BEAN GLUTAREDOXIN	185	514
ATD13875W	GLUTAREDOXIN -LIKE	2.2	S54825 CASTOR BEAN GLUTAREDOXIN	187	507
ATD13880W	GLUTAREDOXIN -LIKE	2.2	S54825 CASTOR BEAN GLUTAREDOXIN	191	508
ATD13885W	GLUTAREDOXIN -LIKE	2.2	S54825 CASTOR BEAN GLUTAREDOXIN	186	504
ATD13890W	GLUTAREDOXIN -LIKE	2.2	S54825 CASTOR BEAN GLUTAREDOXIN	192	503
ATD14000C	UBIQUINOL:CYT.C OXIDOREDUCTASE -LIKE	2.2	Y087296 ALFALFA UBIQUINOL:CYT.C OXIDOREDUCTASE	299	1525
ATD14250W	NAH DEHYDROGENASE -LIKE	2.2	S27171 NEUROSPORA NAH DEHYDROGENASE	122	523
ATD13865C	PYUVATE PHOSPHATE DIKINASE -LIKE	2.3	U02529 DROSOPHILA PYUVATE PHOSPHATE DIKINASE	1446	4631
ATD13820W	DEF PROTEIN	2.3	U27099 ARATH. DEF (CLA1) CHLOROPLAST DEVEL. PROTEIN	3342	4501
ATD13900W	REPLICATION ORIGIN CONTROL PROTEIN -LIKE	3.16	U43416 HUMAN REPLICATION CONTROL PROTEIN 1	855	3829
ATD13420W	CENTROMERE PROTEIN -LIKE	3.16	S28261 HUMAN CENTROMERE PROTEIN	465	7588
ATD14035C	RECA -LIKE	3.19	IQ0738 BACTERIOIDES RECA PROTEIN	92	677
ATD14725W	DNA MISMATCH REPAIR PROTEIN -LIKE	3.19	SP1_DROME DROSOPHILA SPELLCHECKER 1	49	2649
ATD13090C	INDOLE 3 ACETATE GLUCOSYLTRANSE -LIKE	3.26	A54793 MAIZE INDOLE 3 ACETATE GLUCOSYL TRANSE	519	2259
ATD13780C	INDOLE 3 ACETATE GLUCOSYL TRF -LIKE	3.26	A54739 MAIZE INDOLE 3 ACETATE GLUCOSYL TRANSFERASE	681	2484
ATD13785C	INDOLE 3 ACETATE GLUCOSYL TRF -LIKE	3.26	A54739 MAIZE INDOLE 3 ACETATE GLUCOSYL TRANSFERASE	637	2459
ATD13790C	INDOLE 3 ACETATE GLUCOSYL TRF -LIKE	3.26	A54739 MAIZE INDOLE 3 ACETATE GLUCOSYL TRANSFERASE	637	2459
ATD13815C	INDOLE 3 ACETATE GLUCOSYL TRF -LIKE	3.26	A54739 MAIZE INDOLE 3 ACETATE GLUCOSYL TRANSFERASE	575	2262
ATD14410C	GA-20 OXIDASE -LIKE	3.26	U20873 ARATH. GIBBERELLIN 20-OXIDASE GA-5	153	657
ATD14350W	GROWTH REGULATOR -LIKE	3.99	A44226 ARATH. AUXIN-INDEPENDENT GROWTH PROMOTER	1407	4464
ATD13825W	RNA SPLICING ENDONUCLEASE -LIKE	4.1	S53416 YEAST SEN1 PROTEIN	267	554
ATD14685W	ASPARAGINE TRNA LIGASE -LIKE	4.1	B64115 HAEMOPHILUS ASPARAGINE-TRNA LIGASE	404	2147
ATD13300W	RNA POL2 5TH SUBUNIT -LIKE	4.19	B44457 SOYBEAN RNAPOL2 5TH SUBUNIT	138	988
ATD13370C	RNA POL2 5TH SUBUNIT -LIKE	4.19	B44457 SOYBEAN RNAPOL2 5TH LARGEST SUBUNIT	237	821
ATD14580C	TEH SUBUNIT -LIKE	4.1901	Y07595 HUMAN TRANSCRIPTION FACTOR TFIH SUBUNIT	177	517
ATD13030C	HEAT SHOCK TRANSCRIPTION FACTOR -LIKE	4.1904	S25480 PERUVIAN TOMATO HEAT SHOCK TRANSCRIPTION FACTOR	556	4013
ATD13245W	G-BOX BINDING TRANSCRIPTION FACTOR -LIKE	4.1904	U18349 BEAN G-BOX BINDING PROTEIN	140	1308
ATD13310W	CCAAT BOX BINDING FACTOR A SUBUNIT -LIKE	4.1904	C8FA MAIZE CCAAT-BOX BINDING FACTOR	487	758
ATD13670C	CONSTANS -LIKE	4.1904	A56133 ARATH. CONSTANS FLOWERING TIME TXN FACTOR	218	1448
ATD14115W	TRANSCRIPTION FACTOR -LIKE	4.1904	S48041 PARSLEY GC-1 PROTEIN	708	977
ATD14200C	HOMEOBOX -LIKE	4.1904	U41543 C.ELEGANS LIM HOMEOBOX PROTEIN	226	5499
ATD14235C	TRANSCRIPTION ADAPTOR -LIKE	4.1904	A43252 YEAST PROB. TRANSCRIPTION ADAPTOR ADA2	493	2418
ATD14240W	TRANSCRIPTION FACTOR -LIKE	4.1904	U18349 PHASOLUS PHASEOLIN G-BOX BINDING PROTEIN PG2	317	2253
ATD14400C	AP2-DOMAIN TINY -LIKE	4.1904	X04696 ARATH. TINY PROTEIN	144	144
ATD14415W	ATHR1 HOMEOITIC PROTEIN HAT 4	4.1904	S31424 ARATH. HOMEOITIC PROTEIN HAT4	1323	1323
ATD14650C	SCARECROW -LIKE	4.1904	U62978 ARATH. SCARECROW PUTATIVE TRANSCRIPTION FACTOR	404	1834
ATD14765W	HAT 1 HOMEOBOX TRANSCRIPTION FACTOR	4.1904	HAT1_ARATH. HOMEOBOX-LEUCINE ZIPPER HAT1	1331	1331
ATD14780C	EREBP 4 -LIKE	4.1904	D38125 TOBACCO EREBP-4	427	1436
ATD14785W	EREBP-2 -LIKE	4.1904	D38126 TOBACCO EREBP-2 TRANSCRIPTION FACTOR	440	876
ATD14890C	GLABROUS 2 -LIKE	4.1904	A53900 ARATH. HOMEOITIC PROTEIN GL2	487	3104
ATD14910C	HEAT SHOCK TRANSCRIPTION FACTOR 1	4.1904	S52641 ARATH. HEAT SHOCK TRANSCRIPTION FACTOR	3162	3234
ATD14925C	MYB TRANSCRIPTION FACTOR -LIKE	4.1904	S58280 ARATH. MYB TRANSCRIPTION FACTOR	593	2828
ATD14940W	ZN FINGER PROTEIN -LIKE	4.1904	S60325 ARATH. SUPERMAN PROTEIN	216	864
ATD13435C	ATP-DEP. RNA HELICASE -LIKE	4.22	S63453 YEAST SUV3 RNA HELICASE	710	2235
ATD13830C	SPLICING FACTOR -LIKE	4.22	S50096 C.ELEGANS PROB. SPLICING FACTOR	188	2818
ATD13965W	ATP-DEPENDENT RNA HELICASE -LIKE	4.22	S74464 DROSOPHILA DEAD BOX PROTEIN	543	2164
ATD14140W	SPLICING FACTOR -LIKE	4.22	S50096 C.ELEGANS PROB. SPLICING FACTOR	246	1411
ATD14180C	FCA FLOWERING TIME GENE	4.22	Z78992 ARATH. FCA GENE	3547	3547
ATD14340C	ATP-DEPENDENT RNA HELICASE -LIKE	4.22	S64750 ATP-DEPENDENT RNA HELICASE DRS1	715	3239
ATD14365C	RNA HELICASE 1 -LIKE	4.22	A56236 HUMAN RNA HELICASE	2039	4328
ATD13165C	L2 60S RIBOSOMAL PROTEIN -LIKE	5.01	RI2_TOBACCO 60S RIBOSOMAL PROTEIN L2	137	3653
ATD13200C	RIBOSOMAL PROTEIN L41 -LIKE	5.01	M62396 CANDIDA MALITOSA RIBOSOMAL PROTEIN L41	483	753
ATD13545W	L27 RIBOSOMAL PROTEIN -LIKE	5.01	U10046 PEA RIBOSOMAL PROTEIN L27	613	909
ATD14055W	RIBOSOMAL PROTEIN L19 -LIKE	5.01	RSK119 RAT RIBOSOMAL PROTEIN L19	164	544
ATD14365C	RIBOSOMAL PROTEIN L15 -LIKE	5.01	S5490 YEAST RIBOSOMAL PROTEIN L15.E.B	708	977
ATD14790C	RIBOSOMAL PROTEIN L15.E.A -LIKE	5.01	S48502 YEAST RIBOSOMAL PROTEIN L15.E.A	829	1125
ATD14905C	PSII D1 PROTEIN PROCESSING ENZYME -LIKE	6.01	S65416 SPINACH PSII D1 PROCESSING ENZYME	1409	2401
ATD14215C	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE -LIKE	6.07	S45495 S.POMBE PPIA PROTEIN	1114	3648
ATD13440W	PROTEASOME CHAIN C7-1 -LIKE	6.13	S55040 HUMAN PROTEASOME CHAIN HSC7-1	491	954
ATD13560C	SERINE PROTEASE -LIKE	6.13	A55800 MUSK MELON CUCUMISIN PRECURSOR	800	1969
ATD13565C	SERINE PROTEASE -LIKE	6.13	A55800 MUSK MELON CUCUMISIN PRECURSOR	515	1000
ATD13755W	UBIQUITIN DEGRADATION PATHWAY -LIKE	6.13	S59814 YEAST UFD1 PROTEIN	280	3762
ATD14275C	CYSTINE PROTEINASE INHIBITOR -LIKE	6.13	A24464 RICE ORYZASTATIN CYSTINE PROTEINASE INHIBITOR	200	1463
ATD14345C	METALLOENODOPEPTINASE -LIKE	6.13	A41820 SOYBEAN METALLOPEPTINASE	466	1793
ATD14585C	SERINE PROTEASE -LIKE	6.13	A34614 HUMAN PLACENTAL SERINE PROTEASE	274	1135
ATD14790C	UBIQUITIN CARBOXY TERMINAL PROTEINASE -LIKE	6.13	A40085 HUMAN UBIQUITIN C-TERM PROTEASE	396	2193
ATD14265W	SUGAR TRANSPORTER -LIKE	7.07	Z46381 G.C.ELEGANS SUGAR TRANSPORT PROTEIN	491	954
ATD14810C	GLYCEROL-3-PHOSPHATE PERMEASE -LIKE	7.07	HAEMOPHILUS GLYCEROL-3-PHOSPHATE PERMEASE	325	2791
ATD13660W	ABC TRANSPORTER -LIKE	7.25	Z70524 SPIRODELA PDR5-LIKE ABC TRANSPORTER	350	1462
ATD14705W	AQUAPORIN -LIKE	7.99	X59953 HELIANTHUS AQUAPORIN	1056	1184
ATD14120W	CHLOROPLAST PORE PROTEIN -LIKE	8.02	Z73533 PEA CHLOROPLAST PORE PROTEIN	172	1163
ATD13120W	SEC 23 -LIKE	8.07	X97064 HUMAN SEC23 PROTEIN	2060	3814
ATD13930C	SYNAPTOBREVIN -LIKE	8.07	SYBR ARATH. SYNAPTOBREVIN-RELATED PROTEIN	191	816
ATD14080W	SYNTAXIN -LIKE	8.07	L41651 ARATH. SYNTAXIN	654	2209
ATD13490C	HYDROXYPROLINE-RICH GLYCOPROTEIN -LIKE	9.01	S86733 TOBACCO HYDROXYPROLINE RICH GLYCOPROTEIN	911	2525
ATD13625W	CELL WALL PROTEIN -LIKE	9.01	S71558 YEAST CELL WALL MEMBRANE LINKING PROT.	725	2202
ATD13770C	GLYCINE-RICH PROTEIN -LIKE	9.01	X95262 TOMATO TFM5 GENE	194	689
ATD14110W	EXTENSIN -LIKE	9.01	X18186 VIGNA EXTENSIN CLASS 1	178	917
ATD14155W	APG CELL WALL PROTEIN -LIKE	9.01	S21961 ARATH. PROLINE-RICH PROTEIN APG	257	1743
ATD14545C	RYEGRASS ALLERGEN -LIKE	9.01	S18614 RYEGRASS ALLERGEN LGOL P1	300	1433
ATD14645C	TRICHOHYALIN -LIKE	9.01	S28589 RABBIT TRICHOHYALIN	200	2469
ATD13115C	KINESIN -LIKE	9.04	X.LAEVIS KLP2 KINESIN PROTEIN	558	7850
ATD13205W	KINESIN -LIKE	9.04	S54351 C.ELEGANS KINESIN OSM-3	371	4473
ATD13220C	ANKYRIN 2 -LIKE	9.04	S57431 HUMAN ANKYRIN2 LONG FORM	181	4813
ATD14285C	MICROTUBULE-ASSOCIATED PROTEIN 1 LIGHT CHAIN 3 -LIKE	9.04	A53624 MICROTUBULE-ASSOCIATED PROTEIN 1 LIGHT CHAIN 3	167	808

GENE	IDENTITY	FUNCAT.	HOMOLOG	FASTA	SELF FASTA
ATDL4600C	MYOSIN HEAVY CHAIN ATPASE -LIKE	9.04	A24922 RAT EMBRYO MYOSIN HEAVY CHAIN ATPASE	227	2353
ATDL3415W	ACROSIN -LIKE	9.04	S72273 BOVINE ACTIN DEPOLYMERISING PROTEIN	121	1510
ATDL3520C	TUBULIN ALPHA-6 CHAIN	9.04	QJ1597 ARATH. TUBULIN ALPHA-6 CHAIN	2230	2230
ATDL4005W	DYNEIN LIGHT CHAIN -LIKE	9.04	A56444 CHLAMYDOMONAS DYNEIN LIGHT CHAIN	167	495
ATDL3190W	RIBONUCLEOPROTEIN -LIKE	9.13	S40778 XENOPUS RIBONUCLEOPROTEIN	505	1981
ATDL4165C	PHYTOCHROME D	10.01	S46312 ARATH. PHYTOCHROME D	4360	5263
ATDL4205C	CALCIUM SENSOR -LIKE	10.01	Z70783, H C. ELEGANS CA SENSOR PROTEIN	141	724
ATDL3360W	CALMODULIN -LIKE	10.04	L01433 SOYBEAN CALMODULIN	609	693
ATDL3210C	CASEIN KINASE 1	10.04	F88141 ARATH. CASEIN KINASE 1	2309	2309
ATDL3215C	PROTEIN KINASE -LIKE	10.04	S42864 ICE PLANT PROTEIN KINASE	971	3169
ATDL3280C	SPS-1 KINASE -LIKE	10.04	HL B1080 YEAST SPS-1 SPORE SPECIFIC KINASE	443	2327
ATDL3330C	SNF1 KINASE -LIKE	10.04	A53467 WHEAT SNF1 HOMOLOG	770	2075
ATDL3430W	PROTEIN KINASE -LIKE	10.04	S29851 SOYBEAN PROTEIN KINASE 6	472	1833
ATDL3865W	PROTEIN KINASE -LIKE	10.04	S49313 SLIME MOULD PROTEIN KINASE	475	1050
ATDL4210W	AMP-ACTIVATED PROTEIN KINASE -LIKE	10.04	U24811 RAT AMP-ACTIVATED PROTEIN KINASE BETA SUBUNIT	381	1961
ATDL4255W	PROTEIN KINASE -LIKE	10.04	H54024 HUMAN CDC-2 RELATED PROTEIN KINASE	104	607
ATDL4515C	PROTEIN KINASE -LIKE	10.04	S56143 S. POMBE PROTEIN KINASE HSK1	164	3806
ATDL4855W	CASEIN KINASE 2 BETA CHAIN	10.04	S47968 ARATH. CASEIN KINASE 2 BETA CHAIN	1444	1444
ATDL4863W	PROTEIN KINASE -LIKE	10.04	S38326 ARATH. PROTEIN KINASE	866	1820
ATDL3750W	PROTEIN PHOSPHATASE -LIKE	10.041	U38193 ORYCTOLAGUS PROTEIN PHOSPHATASE	1813	4517
ATDL3905C	COP9	10.99	A54842 ARATH. COP9	1016	1016
ATDL3990W	PR1-1 G-BETA PROTEIN	10.99	S49820 ARATH. PRL1 PROTEIN	1806	1806
ATDL3000W	DISEASE RESISTANCE GENE -LIKE	11.01	U24455 SOLANUM PIMP. CF-2.2	1131	4278
ATDL3225C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	1392	9717
ATDL3345C	DISEASE RESISTANCE PROTEIN -LIKE	11.01	A54809 ARATH. RPP2 DISEASE RESISTANCE PROTEIN	152	592
ATDL4460C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	4326	6448
ATDL4470C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	387	1246
ATDL4475C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	3495	6010
ATDL4480C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	2567	258
ATDL4490C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	5065	6675
ATDL4495C	RPP5 -LIKE (FRAGMENT)	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	408	753
ATDL4500C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	2723	3568
ATDL4505C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	5106	6705
ATDL4510C	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	2354	5261
ATDL4525W	DOWNY MILDEW RESISTANCE PROTEIN RPP5 -LIKE	11.01	G2109275 ARATH. DOWNY MILDEW RESISTANCE PROTEIN RPP5	1286	4556
ATDL3070W	MAJOR LATEX PROTEIN TYPE 1 -LIKE	11.02	X91914 ARATH. MAJOR LATEX HOMOLOG TYPE 2	641	763
ATDL3155C	HSP-70 RELATED PROTEIN -LIKE	11.05	U41538, H C. ELEGANS HSP70 -LIKE PROTEIN	27	1878
ATDL3375W	HEAT SHOCK PROTEIN -LIKE	11.05	L35272 SOYBEAN HEAT SHOCK PROTEIN	2043	3482
ATDL3385W	EARLY LIGHT INDUCED PROTEIN (ELIP) -LIKE	11.05	S71560 SUNEFLOWER ELIP SDI-1 DROUGHT INDUCED PROTEIN	475	1548
ATDL3995W	DROUGHT-INDUCED PROTEIN D121 -LIKE	11.05	S14179 ARATH. DROUGHT-INDUCED PROTEIN D121	318	404
ATDL4135W	CYSTEINE PROTEINASE -LIKE	11.05	DN0718 ARATH. DROUGHT-IND. CYSTEINE PROTEINASE	1377	1888
ATDL4295C	HEAT SHOCK PROTEIN 21 -LIKE	11.05	S16004 PETUNIA HSP 21	110	1128
ATDL4300C	HEAT SHOCK PROTEIN 21 -LIKE	11.05	S65049 SOYBEAN HSP 23.9 PRECURSOR	125	3721
ATDL4615C	GTP-BINDING RAB2A -LIKE	11.05	Z73937 LOTUS JAPONICUS RAB2A	1004	2248
ATDL4620C	GTP-BINDING RAB2A -LIKE	11.05	Z73937 LOTUS JAPONICUS RAB2A	1010	2248
ATDL4800C	GTP-BINDING RAB1C -LIKE	11.05	Z73932 LOTUS JAPONICUS RAB1C	710	1011
ATDL4835W	HEAT SHOCK TRANSCRIPTION FACTOR 21 -LIKE	11.05	S59537 SOYBEAN HEAT SHOCK TRANSCRIPTION FACTOR 21	127	1759
ATDL3055C	SELENIUM BINDING PROTEIN -LIKE	11.06	S27828 MOUSE HEPATIC SELENIUM-BINDING PROTEIN	1609	2542
ATDL3060C	SELENIUM BINDING PROTEIN -LIKE	11.06	S27828 MOUSE HEPATIC SELENIUM-BINDING PROTEIN	1211	5455
ATDL3015C	PREDICTED	12	L_41438, D WALLACE DERMAL SARCOMA VIRUS GAG PROTEIN	98	256
ATDL3020C	PREDICTED	12	A49282 SUGAR BEET YELLOW VIRUS FUSION PROTEIN 1A/1B	138	2254
ATDL3035W	PREDICTED	12	IS2418 HUMAN CYTOCHROME P450	98	2593
ATDL3050C	PREDICTED	12	TRANSLATION INITIATION FACTOR IF-2 PRECURSOR	81	542
ATDL3075C	AMP-BINDING PROTEIN -LIKE	12	Z72151 BRASSICA NAPUS AMP BINDING PROTEIN	2982	3462
ATDL3085W	PREDICTED	12	G1374712 PROTON-COUPLED PEPTIDE TRANSPORTER PEPT	105	5404
ATDL3100W	PREDICTED	12	U66560, C MYCOBACTERIUM AVIUM EMBB	98	1134
ATDL3125C	MEMBRANE PROTEIN -LIKE	12	U08285 TOBACCO SALT-INDUCED MEMBRANE PROTEIN	123	2236
ATDL3135C	MEMBRANE PROTEIN -LIKE	12	U08285 TOBACCO SALT-INDUCED MEMBRANE PROTEIN	212	2938
ATDL3140C	PREDICTED	12	S52076 SLIME MOULD PROTEIN KINASE	102	3142
ATDL3150W	PREDICTED	12	A43444 HUMAN TAU PROTEIN	105	1518
ATDL3170C	PREDICTED	12	S17286 DROSOPHILA PER PROTEIN	83	2308
ATDL3175W	PREDICTED	12	TIH18 BARLEY TRYPSIN INHIBITOR	12	58
ATDL3185C	PREDICTED	12	S29274 PHBC, CHRV1 POLYHYDROXYBUTYRATE POLYMERASE	96	2524
ATDL3195C	PREDICTED	12	MMMSB1 MOUSE LAMININ CHAIN B1 PRECURSOR	119	5907
ATDL3230W	PREDICTED	12	A36985 S. POMBE P TYPE MATING FACTOR	101	978
ATDL3235W	PREDICTED	12	U00111 CHICKEN NFKB	125	3453
ATDL3240W	PREDICTED	12	A37867 HUMAN FRB	144	3279
ATDL3265W	PREDICTED	12	A41541 RAT ADENYLATE CYCLASE	83	744
ATDL3285C	PREDICTED	12	D50867 HALOCYNTNTHA TROPONIN T	101	1759
ATDL3290W	PREDICTED	12	S57929 BOVINE PHOSPHATIDYL CHOLINE TRANSFER PROTEIN	102	2375
ATDL3295C	PREDICTED	12	U09782 ARGOPECTEN MYOSIN HEAVY CHAIN	118	8317
ATDL3305C	PREDICTED	12	S05518 CHICKEN LAMIN B-1	98	612
ATDL3315C	IAA7 PROTEIN -LIKE	12	S58494 ARATH. IAA7 PROTEIN	875	939
ATDL3320W	AUXIN-RESPONSIVE PROTEIN IAA1	12	AX11 ARATH. AUXIN-INDUCED PROTEIN	826	827
ATDL3335W	PREDICTED	12	S45416 TEL1 PROTEIN YEAST	119	2618
ATDL3340W	PREDICTED	12	S74455 SYNECHOCYSTIS ABC TRANSPORTER PROTEIN	101	2874
ATDL3350C	PREDICTED	12	S34961 RAT SYNAPTIC VESICLE PROTEIN 2 FORM B	92	1697
ATDL3365C	PREDICTED	12	S00485 PLASMODIUM GENE 11-1 PROTEIN PRECURSOR	181	2846
ATDL3400C	PREDICTED	12	S67359 YEAST MNE1 GENE	117	277
ATDL3405W	PREDICTED	12	S19586 RAT N-ME-D-ASPARTATE RECEPTOR PROTEIN	130	523
ATDL3410C	PREDICTED	12	A56678 DROSOPHILA YEMANUCLEIN-ALPHA	96	2257
ATDL3425C	PREDICTED	12	S39162 HUMAN CREB BINDING PROTEIN	92	3031
ATDL3430W	PREDICTED	12	L29389 YEAST FUN12P	121	958
ATDL3450C	PREDICTED	12	M92439 HUMAN LEUCINE-RICH PROTEIN	173	3677
ATDL3455W	PREDICTED	12	U47087 CARROT PATHOGENESIS-RELATED PROTEIN	87	741
ATDL3460C	PREDICTED	12	D45163 HALOCYNTNTHA MYOSIN HEAVY CHAIN	127	2524
ATDL3465W	PREDICTED	12	S2946 RHODOSPIRILLUM RUBRUM PROTEIN	90	2526
ATDL3470W	PREDICTED	12	L39769, E STREPTOCOCCUS PLASMID pIP501 GENES	88	881
ATDL3475W	PREDICTED	12	SECE_THEMA PREPROTEIN TRANSLOCASE	124	786
ATDL3505W	PREDICTED	12	G64369 M.JANNA SCHII SURE SURVIVAL PROTEIN	113	1362
ATDL3515W	PREDICTED	12	KS260 HUMAN PROGESTERONE MEMBRANE BINDING PROTEIN	143	3999
ATDL3525W	PREDICTED	12	U35238 ORYCTOLAGUS NA CHANNEL	106	3972
ATDL3530C	PREDICTED	12	TVHUBF HUMAN PROTEIN KINASE B-RAF	92	737
ATDL3535C	PREDICTED	12	RXRb MOUSE RETINOIC ACID RECEPTOR RXR-BETA	103	5008
ATDL3570C	PREDICTED	12	C84212 MYCOPLASMA GENITALIUM PGIB	95	2093
ATDL3580C	PREDICTED	12	S14422 C. ELEGANS ENDOPROTEINASE	135	4670
ATDL3605W	PREDICTED	12	F47021 ERWINIA SP. OUTH PECTIC ENZYME SECRETION	94	831
ATDL3615C	PREDICTED	12	WMNV 49 40.9K AUTOGRAPHIA NUCLEAR POLYHEDROSIS VIRUS	96	906
ATDL3620C	PREDICTED	12	X05285 DROSOPHILA FIBRILLARIN	119	481
ATDL3640C	PREDICTED	12	S59811 YEAST PEP7	81	604
ATDL3740C	PREDICTED	12	X95343 TOBACCO HSR201 PROTEIN	247	2126
ATDL3745C	PREDICTED	12	X95343 TOBACCO HSR201 PROTEIN	237	2090
ATDL3795W	PREDICTED	12	A11369 CABBAGE S RECEPTOR KINASE	86	1363
ATDL3810W	PREDICTED	12	A40393 MOUSE CYSTIC FIBROSIS CONDUCTANCE RECEPTOR	130	2378
ATDL3845W	PREDICTED	12	IS1270 POEPHILA MYELIN PROTEOLIPID PROTEIN	86	718
ATDL3850W	PREDICTED	12	U52866, C RHIZOBIUM CCMB CYTOCHROME ASSEMBLY	93	913
ATDL3855W	PREDICTED	12	S74647 SYNECHOCYSTIS SPORE MATURATION PROTEIN B	97	885
ATDL3860C	PREDICTED	12	L76937 HUMAN WERNER SYNDROME GENE	19	605
ATDL3895W	PREDICTED	12	A44357 SLIME MOULD DYNEIN HEAVY CHAIN	90	739
ATDL3900W	MEMBRANE PROTEIN -LIKE	12	U08285 TOBACCO MEMBRANE ASSOC.SALT-INDUCED PROTEIN	146	2972
ATDL3910C	PREDICTED	12	B37237 XENOPUS PROTEIN KINASE C	109	2237
ATDL3935W	PREDICTED	12	G155761 NARCELARIA MYOSIN II HEAVY CHAIN	112	517
ATDL3940C	IAF86 GTP-BINDING PROTEIN -LIKE	12	PSIAP86A, 1 PEA IAF86 GTP-BINDING PROTEIN	405	2260
ATDL3950W	PREDICTED	12	A38713 SEA URCHIN KINESIN HEAVY CHAIN	108	2807
ATDL3955C	PREDICTED	12	U18792 BARESSIA GLUTAMATE DEP. CARBAMOYL P SYNTH.	90	1178
ATDL3960W	PREDICTED	12	U20449 PARAMECIUM DYNEIN HEAVY CHAIN	89	3278
ATDL4025W	ZINC-FINGER PROTEIN -LIKE	12	Z72511, C C. ELEGANS ZN FINGER PROTEIN	195	2310
ATDL4065W	PREDICTED	12	A40253 YEAST ACIDIC NUCLEAR PROTEIN SPT5	145	4425
ATDL4070W	PREDICTED	12	RPOB, CYANOPHORA DNA-DIRECTED RNA POL. BETA CHAIN	103	1367
ATDL4085W	PREDICTED	12	S74857 E. COLI OUTER MEMBRANE PROTEIN	95	1312
ATDL4090W	PREDICTED	12	U31777 RAT ATROPHIN-1 PROTEIN	89	905
ATDL4095W	PREDICTED	12	B33862 E.COLI REGULATORY PROTEIN HYD	131	2974
ATDL4100C	PREDICTED	12	ODZJ1 BRADYRHIZOBIUM CYTOCHROME-C OXIDASE	109	3298
ATDL4125C	PREDICTED	12	G64419 M. JANNASCHII SERINE AMINOTRANSFERASE	92	2310
ATDL4130C	PREDICTED	12	S23662 E.COLI CYTOSINE DEAMINASE	83	1494
ATDL4160C	GLYCINE RICH PROTEIN -LIKE	12	QJ1060 ARATH. GLYCINE-RICH PROTEIN	155	207
ATDL4190W	PREDICTED	12	A42111 ENTEROCOCCUS NAPA Na/H EXCHANGING PROTEIN	84	1375
ATDL4220C	PREDICTED	12	S42629 RABBIT KERATIN	113	386
ATDL4225W	MEMBRANE PROTEIN -LIKE	12	U08285 TOBACCO MEMBRANE ASSOC.SALT-INDUCIBLE PROT.	325	3832
ATDL4233C	PREDICTED	12	X92429 STREPTOMYCES N-METHYL TRANSFERASE	98	916
ATDL4245C	PREDICTED	12	S31336 KLUVERO MYCES LET1 PROTEIN	185	2827

GENE	IDENTITY	FUN.CAT.	HOMOLOG	FASTA	SELF FASTA
ATDL4260C	MEMBRANE PROTEIN -LIKE	12	U08285 TOCACCO MEMBRANE-ASSOC. SALT-INDUCIBLE PROT.	120	2378
ATDL4290W	PREDICTED	12	U09274 CARCINUS NA/H EXCHANGER	122	3848
ATDL4305C	PREDICTED	12	L47741 PICEA MITOCHONDRIAL HSP 23.5	145	5215
ATDL4310W	PREDICTED	12	A41098 RAT CA CHANNEL ISOFORM A	100	2389
ATDL4330C	PREDICTED	12	S02035 DROSOPHILA PER PROTEIN	107	945
ATDL4335C	PREDICTED	12	U43629 BEET MEMBRANE PROTEIN	174	2907
ATDL4355W	PREDICTED	12	D85904 MOUSE APG-2	306	1323
ATDL4360W	PREDICTED	12	A40718 HUMAN HOST CELL FACTOR C1 PRECURSOR	115	1848
ATDL4420C	PREDICTED	12	A29356 KIDNEY BEAN HYDROXYPROLINE RICH PROTEIN	158	2672
ATDL4430C	PREDICTED	12	S28261 HUMAN CENTROMERE PROTEIN E	97	2866
ATDL4440W	PREDICTED	12	U26024 BOVINE NUCLEAR ANTIGEN	152	1826
ATDL4445W	MEMBRANE PROTEIN -LIKE	12	U02825 TOBACCO MEMBRANE ASSOC. SALT-IND. PROT	148	4334
ATDL4450W	PREDICTED	12	A28755 N. CRASSA UBQ CYTO. C REDUCTASE	115	1499
ATDL4520C	PREDICTED	12	A26099 SOYBEAN GLYCINE-RICH CELL WALL PROTEIN	132	480
ATDL4530C	PREDICTED	12	A45990 RABBIT TRIADIN PROTEIN	132	3046
ATDL4535W	PREDICTED	12	B42477 E.COLI PHOSPHOTRANSFERASE SYSTEM ENZYME II	90	1577
ATDL4560C	PREDICTED	12	A34840 XENOPUS HRNP 1a	133	1440
ATDL4565C	PREDICTED	12	S40507 RUMEN FUNGUS ENDOGLUCANASE	94	1620
ATDL4590C	PREDICTED	12	A48805 MOUSE INSULIN-LIKE GROWTH FACTOR I	115	1722
ATDL4600C	PREDICTED	12	S75000 SYNECHOCYSTIS MG CHELATASE SUBUNIT	104	3630
ATDL4605C	PREDICTED	12	U29535 C. ELEGANS SEX MUSCLE ABNORMAL PROTEIN 5	132	6666
ATDL4610W	PREDICTED	12	A35548 RHIZOBIUM MELILOTI adhB PROTEIN	107	1991
ATDL4635W	PREDICTED	12	A37353 XENOPUS MEMBRANE PROTEIN	91	1954
ATDL4655C	PREDICTED	12	H0280 HUMAN 160K GOLGI ANTIGEN	119	1479
ATDL4660W	PREDICTED	12	U12925 A. CERATIS MITOCHONDRIAL NADH DEH'ASE	99	3073
ATDL4675C	PREDICTED	12	U18197 HUMAN ATP-CITRATE LYASE	100	1262
ATDL4695C	PREDICTED	12	S64942 YEAST PROBABLE MEMBRANE PROTEIN	111	8955
ATDL4710W	PREDICTED	12	YG4578 BACILLUS BREVIS TYROCIDINE SYNTHASE	89	1806
ATDL4740W	HUMAN DNA-BINDING PROTEIN 5 -LIKE	12	S2650 HUMAN DNA-BINDING PROTEIN 5	188	2114
ATDL4745C	PREDICTED	12	S51330 HUMAN FLAVIN-CONTAINING MONOOXYGENASE	95	1389
ATDL4755W	PREDICTED	12	A38712 HUMAN FIBRILLARIN	111	1044
ATDL4805W	PREDICTED	12	I54222 MOUSE HOUSEKEEPING PROTEIN	84	950
ATDL4815W	PREDICTED	12	S51232 PEA OVARY PROTEIN	188	2114
ATDL4820C	PREDICTED	12	A64251 MYCOPLASMA GLUTAMATE-TRNA LIGASE	106	3767
ATDL4825C	TEGT -LIKE	12	S42069 RAT TEGT PROTEIN	359	1345
ATDL4830C	PREDICTED	12	A48998 MOUSE NUCLEOLAR PROTEIN	115	2690
ATDL4840C	TRP-185 -LIKE	12	S62356 HUMAN TRP-185 PROTEIN	291	7303
ATDL4845W	PREDICTED	12	S60465 C.ELEGANS DOM3 PROTEIN	149	5793
ATDL4850C	PREDICTED	12	A33638 MOUSE ANION EXCHANGER AE3	117	921
ATDL4860W	MAJOR SPERM PROTEIN -LIKE	12	U23515 F.C. ELEGANS MAJOR SPERM PROTEIN	188	1357
ATDL4870C	PREDICTED	12	C5324 MAIZE GLOBULIN-10	89	3073
ATDL4875C	PREDICTED	12	S68451 HUMAN APOPTOSIS-INHIBITOR XIAP	122	1403
ATDL4895C	PREDICTED	12	A49465 BOVINE COATOMER ZETA CHAIN	95	1379
ATDL4915W	PREDICTED	12	A24302 RABBIT GLYCOGEN PHOSPHORYLASE	92	1252
ATDL4935C	PREDICTED	12	U17015 PSEUDOMONAS CEPHALOSPORIN CYCLASE	112	1360
ATDL3025C	HYPOTHETICAL	13	79639 C.ELEGANS HYPOTHETICAL PROTEIN F54E4.1	114	3149
ATDL3040W	HYPOTHETICAL	13	Z. 514_B.C.ELEGANS HYPOTHETICAL PROTEIN	240	1330
ATDL3045C	HYPOTHETICAL	13			587
ATDL3065C	HYPOTHETICAL	13	U15181_AT MYCOBACTERIUM HYPOTHETICAL PROTEIN	109	1262
ATDL3130W	HYPOTHETICAL	13	D46006 SYNECHOCYSTIS HYPOTHETICAL PROTEIN	113	6613
ATDL3160C	HYPOTHETICAL	13	U41548_C.C.ELEGANS HYPOTHETICAL PROTEIN	323	2402
ATDL3180W	HYPOTHETICAL	13	S25990 LIVERWORT HYPOTHETICAL PROTEIN	105	4025
ATDL3250C	HYPOTHETICAL	13	S55204 HYPOTHETICAL YEAST PROTEIN	88	762
ATDL3395C	HYPOTHETICAL	13	S53039 YEAST HYPOTHETICAL PROTEIN YMR009w	341	4348
ATDL3500C	HYPOTHETICAL	13	E24548_C.ELEGANS HYPOTHETICAL	153	5124
ATDL3540C	HYPOTHETICAL	13	H6451 METHANOCOCCLUS HYPOTHETICAL PROTEIN MJEC508	103	1911
ATDL3550W	HYPOTHETICAL	13			791
ATDL3555W	HYPOTHETICAL	13	Z54342_0_C.ELEGANS F36C1.1	138	3132
ATDL3575W	HYPOTHETICAL	13	S44609_C.ELEGANS C02F.7	140	2855
ATDL3585C	HYPOTHETICAL	13	S41011 HYPOTHETICAL PROTEIN ZK757.1_C. ELEGANS	168	3555
ATDL3590W	HYPOTHETICAL	13	S47183 STREPTOMYCES GRISEUS HYP. PROTEIN	453	8613
ATDL3630W	HYPOTHETICAL	13			148
ATDL3635W	HYPOTHETICAL	13	Z77655_G_C56A3.1_C.ELEGANS HYPOTHETICAL PROTEIN	148	11666
ATDL3645C	HYPOTHETICAL	13	A34043 POLYCHAETE HYPOTHETICAL PROLINE RICH PROTEIN	193	3063
ATDL3665C	HYPOTHETICAL	13	YR47_CAEEL HYPOTHETICAL PROTEIN	94	2693
ATDL3760W	HYPOTHETICAL	13	S51583 ARATH HYPOTHETICAL PROTEIN HYP1	801	3843
ATDL3775W	HYPOTHETICAL	13	S44609_C.ELEGANS C02F.7 HYPOTHETICAL PROTEIN	445	3684
ATDL3800W	OBP 33 PEP PROTEIN -LIKE	13	S71213 ARATH. OBP33 PEP PROTEIN	893	1389
ATDL3905C	HYPOTHETICAL	13	G1469195 HUMAN KIAA0136 PROTEIN	151	5028
ATDL3915C	HYPOTHETICAL	13	S10911 CARROT HYPOTHETICAL PROTEIN	105	834
ATDL3920C	HYPOTHETICAL	13	D64366 M.JANASCHII HYPOTHETICAL PROTEIN	106	3195
ATDL3925W	HYPOTHETICAL	13	S63230 YEAST HYPOTHETICAL PROTEIN YP1211w	267	368
ATDL3945C	HYPOTHETICAL	13	S55101 YEAST HYPOTHETICAL PROTEIN YMR219w	117	2268
ATDL3980W	HYPOTHETICAL	13	YY06_HUMAN HYPOTHETICAL MYELOID CELL LINE 6 PROTEIN	394	8268
ATDL3985W	HYPOTHETICAL	13	G473941_HUMAN ORF	727	460
ATDL4040C	HYPOTHETICAL	13			460
ATDL4060C	HYPOTHETICAL	13	U23176_C.ELEGANS COSMID F21H12	83	438
ATDL4075C	HYPOTHETICAL	13	U00051_G.C.ELEGANS COSMID F42G9	188	3232
ATDL4080C	HYPOTHETICAL NON-LTR	13	Z70270_G.C.ELEGANS C53K.7	107	1979
ATDL4185W	HYPOTHETICAL	13	Z3562_C.C.ELEGANS HYPOTHETICAL PROTEIN	484	5906
ATDL4230W	HYPOTHETICAL	13	D79987 HUMAN KIAA0165 HYPOTHETICAL PROTEIN	117	1060
ATDL4270C	HYPOTHETICAL	13	S50446 YEAST HYPOTHETICAL PROTEIN YELO13w	165	2093
ATDL4280W	HYPOTHETICAL	13			521
ATDL4315C	HYPOTHETICAL	13	S46810 YEAST HYPOTHETICAL PROTEIN YHR076W	124	2104
ATDL4380W	HYPOTHETICAL	13	S64051 YEAST HYPOTHETICAL PROTEIN YGLO47W	170	851
ATDL4535W	HYPOTHETICAL	13			386
ATDL4550C	HYPOTHETICAL NON-LIKE	13	S75438 SYNECHOCYSTIS HYPOTHETICAL PROTEIN	358	1124
ATDL4570W	HYPOTHETICAL	13	S74454 SYNECHOCYSTIS HYPOTHETICAL PROTEIN SLR1485	331	2382
ATDL4580W	HYPOTHETICAL	13	Z69883_D.C.ELEGANS HYPOTHETICAL PROTEIN	94	726
ATDL4595C	HYPOTHETICAL	13	Z80220_C.C.ELEGANS T08G.1	189	8294
ATDL4670W	HYPOTHETICAL	13	I57997 MOUSE HYPOTHETICAL CA BINDING PROTEIN	429	1108
ATDL4680W	HYPOTHETICAL	13	I54209 HUMAN HYPOTHETICAL PROTEIN	83	541
ATDL4690C	HYPOTHETICAL	13			271
ATDL4700C	HYPOTHETICAL	13	I51116 SEA LAMPREY HYPOTHETICAL PROTEIN NF-180	136	7495
ATDL4735W	HYPOTHETICAL	13	K_1441 YEAST HYPOTHETICAL PROTEIN	265	1340
ATDL4750C	HYPOTHETICAL	13	C64015 HAEMOPHILUS HYPOTHETICAL PROTEIN	106	2391
ATDL4795W	HYPOTHETICAL	13	U53154_K.C.ELEGANS HYPOTHETICAL PROTEIN	155	1621
ATDL4885W	HYPOTHETICAL	13	Z50875_B.C.ELEGANS HYPOTHETICAL PROTEIN	85	1803
ATDL4930W	HYPOTHETICAL	13	D26067 HUMAN ORF41	530	1353
ATDL3270W	RETROELEMENT POLYPROTEIN TAI-3 LIKE	14	S05465 ARATH. RETROELEMENT LTR	765	8744
ATDL3275W	RETROELEMENT TAI1-1 LIKE	14	E248476 NON-LTR RETROTRANSPONSON	348	1702
ATDL3835C	RETROTRANSPONSON NON-LTR	14	S65812 ARATH. RETROTRANSPONSON TAI1-1 REVXASE	815	4838
ATDL3840C	RETROTRANSPONSON FINGER PROTEIN	14	S65811 ARATH. RETROTRANSPONSON TAI1-1 FINGER PROT.	177	3204
ATDL3970C	RETROELEMENT NON-LTR	14	S23315 RETROVIRUS RELATED PROTEIN TAI-2	238	1145
ATDL4045W	RETROTRANSPONSON LTR	14	U12626 MAIZE HOPSOTCH RETROTRANSPONSON	1112	9689
ATDL4050W	RETROTRANSPONSON LTR	14	U12626 MAIZE HOPSOTCH RETROTRANSPONSON	585	1659
ATDL4465C	COPIA-LIKE LTR RETROTRANSPONSON	14	G531389 MAIZE COPIA-LIKE TRANSPONSON HOPSOTCH	1459	7098
ATDL4485C	LTR RETROTRANSPONSON	14	JN0791 TRITICUM YEAST RETROTRANSPONSON PROTEIN	720	1458
ATDL4760C	LTR RETROTRANSPONSON	14	U12626 MAIZE HOPSOTCH RETROELEMENT PROTEIN	1252	7148
ATDL3600C	CYTOCHROME P450 -LIKE	20.1	S71663 PEA CYTOCHROME P450	2118	2948
ATDL3695C	CYTOCHROME P450 -LIKE	20.1	S55379 ARATH. CYTOCHROME P450	490	2569
ATDL3700W	CYTOCHROME P450 -LIKE	20.1	S55379 ARATH. CYTOCHROME P450	317	1558
ATDL3710C	CYTOCHROME P450 -LIKE	20.1	S62899 SOYBEAN CYTOCHROME P450	977	2617
ATDL3720W	CYTOCHROME P450 -LIKE	20.1	S62899 SOYBEAN CPY93 A1 CYTOCHROME P450	829	2574
ATDL3725W	CYTOCHROME P450 -LIKE	20.1	S62899 SOYBEAN CPY93 A1 CYTOCHROME P450	1016	2701
ATDL3735W	CYTOCHROME P450 -LIKE	20.1	S62899 SOYBEAN CPY93 A1 CYTOCHROME P450	784	2489
ATDL4175W	PEROXIDASE -LIKE	20.1	I37790 STYLOKANTHES CATIONIC PEROXIDASE	548	1698
ATDL4195C	DIOXYGENASE -LIKE	20.1	L42466 PICEA ETHYLENE FORMING ENZYME	475	1217
ATDL4880W	PEROXIDASE -LIKE	20.1	L36158 MEDICAGO PEROXIDASE	789	1610
ATDL3715C	LUPUL SYNTHASE -LIKE	20.2	ATU49919_1 ARATH.LUPUL SYNTHASE	1309	4493
ATDL3730C	LUPUL SYNTHASE -LIKE	20.2	ATU49919_1 ARATH.LUPUL SYNTHASE	787	3274
ATDL3975C	SESQUITERPENE CYCLASE -LIKE	20.2	U20187 HYOSCAMUS VETISPIADIENE SYNTHASE	582	918
ATDL4390W	LIMONENE CYCLASE -LIKE	20.2	A48863 SPEARMINT LIMONENE CYCLASE	1189	3020
ATDL4395W	LIMONENE CYCLASE -LIKE	20.2	A48863 SPEARMINT LIMONENE CYCLASE	1041	5230
ATDL4150W	MYRONSINE-ASSOCIATED PROTEIN -LIKE	20.6	U39289 BRASSICA MYRONSINE-ASSOCIATED PROTEIN	128	960
ATDL3510W	COPPER AMINE OXIDASE -LIKE	20.99	C44239_PEA CU-AMINE OXIDASE	1684	7228
ATDL3595W	HYDROXYNITRILE LYASE -LIKE	20.99	S53311 SORGHUM HYDROXYNITRILE LYASE	762	2134
ATDL4370C	S-HYDROXYNITRILE LYASE -LIKE	20.99	U40402 HEVEA HYDROXYNITRILE LYASE	346	1325

Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12

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FKBP12, a *cis-trans* prolyl isomerase that binds the immunosuppressants FK506 and rapamycin, is ubiquitously expressed and interacts with proteins in several intracellular signal transduction systems¹. Although FKBP12 interacts with the cytoplasmic domains of type I receptors of the transforming growth factor- β (TGF- β) superfamily *in vitro*, the function of FKBP12 in TGF- β superfamily signalling is controversial²⁻⁶. FKBP12 also physically interacts stoichiometrically with multiple intracellular calcium release channels including the tetrameric skeletal muscle ryanodine receptor (RyR1)^{7,8}. In contrast, the cardiac ryanodine receptor, RyR2, appears to bind selectively the FKBP12 homologue, FKBP12.6 (refs 9, 10). To define the functions of FKBP12 *in vivo*, we generated mutant mice deficient in FKBP12 using embryonic stem (ES) cell technology. FKBP12-deficient mice have normal skeletal muscle but have severe dilated cardiomyopathy and ventricular septal defects that mimic a human congenital heart disorder, noncompaction of left ventricular myocardium^{11,12}. About 9% of the mutants exhibit exencephaly secondary to a defect in neural tube closure. Physiological studies demonstrate that FKBP12 is dispensable for TGF- β -mediated signalling, but modulates the calcium release activity of both skeletal and cardiac ryanodine receptors.

FKBP12 exons 3 and 4 encode functional domains involved in FK506 and rapamycin binding¹³, *cis-trans* prolyl isomerase activity¹³, and TGF- β family type I receptor binding^{2,3}. To generate a null mutation, a targeted deletion (*fkbp12^{ml}*) of these key exons was generated using ES cell technology (Fig. 1a). Heterozygous

(*fkbp12^{ml}/+*) mice were viable and fertile and were intercrossed to obtain FKBP12-deficient (*fkbp12^{ml}/fkbp12^{ml}*) mice (Fig. 1b). Genotype analysis of 376 F₂ C57BL/6J/129SvEv hybrid and 181 F₂ 129SvEv inbred offspring at weaning demonstrated only 8 viable FKBP12-deficient mice. The distribution of genotypes at embryonic day 14.5 (E14.5) (88 wild-type (25%), 177 heterozygotes (51%), and 84 homozygotes (24%)) and at E18.5 (220 wild-type (30%), 390 heterozygote (53%), and 125 homozygotes (17%)) suggested that the majority of the mutant mice died between E14.5 and birth.

To determine the causes of this embryonic and neonatal lethality, embryos derived by caesarean section were examined morphologically and histologically. The majority of FKBP12-deficient mutants died between E14.5 and birth because of severe dilated cardiomyopathy and ventricular septal defects (VSD). At E18.5, the majority of the FKBP12-deficient mice gasped for breath, but continued to exhibit pallor despite normal haematocrit, and demonstrated a rapid demise suggesting a failure of perfusion. Some of these E18.5 FKBP12-deficient mice were oedematous, and the majority had dramatically enlarged hearts because of four-chamber dilation (Fig. 2a, b). The FKBP12-deficient heart weight (13.4 ± 0.44 mg) was greatly increased compared to controls (8.14 ± 0.38 mg) ($P < 0.0005$). Detailed histological analysis revealed multiple abnormal anatomical structures in FKBP12-deficient hearts (Fig. 2c, d), which resembled the human congenital heart disorder, noncompaction of left ventricular myocardium, which is often accompanied by ventricular septal defects^{11,12}. In E14.5 control hearts (Fig. 2c), the ventricular walls were of roughly equal thickness and trabeculated. However, the FKBP12-deficient hearts at E14.5 and E18.5 exhibited prominent ventricular septal defects, increased cavity diameters, thinner left ventricular walls, hypertrophic trabeculae, and deep intertrabecular recesses (Fig. 2d and data not shown), indicating that the condensation of myocardium is disrupted in the FKBP12-deficient hearts. This myocardial noncompaction appears to be independent of the ventricular septal defect because other mouse mutants with ventricular septal defects do not have myocardial noncompaction^{14,15}. Consistent with these defects, *in situ* hybridization revealed that FKBP12 is expressed at high levels throughout the embryonic heart including endocardium, myocardium, and septum (Fig. 2e). Lastly, consistent with this cardiac dysfunction, severe haemorrhage and necrosis were observed in the livers of many FKBP12-deficient mutants (Fig. 2f, g), mimicking the liver centrilobular necrosis seen in passive congestive heart failure patients.

In addition to the cardiac defects, 9% (8/84) of the E14.5 and 4% (5/125) of the E18.5 FKBP12-deficient embryos had exencephaly

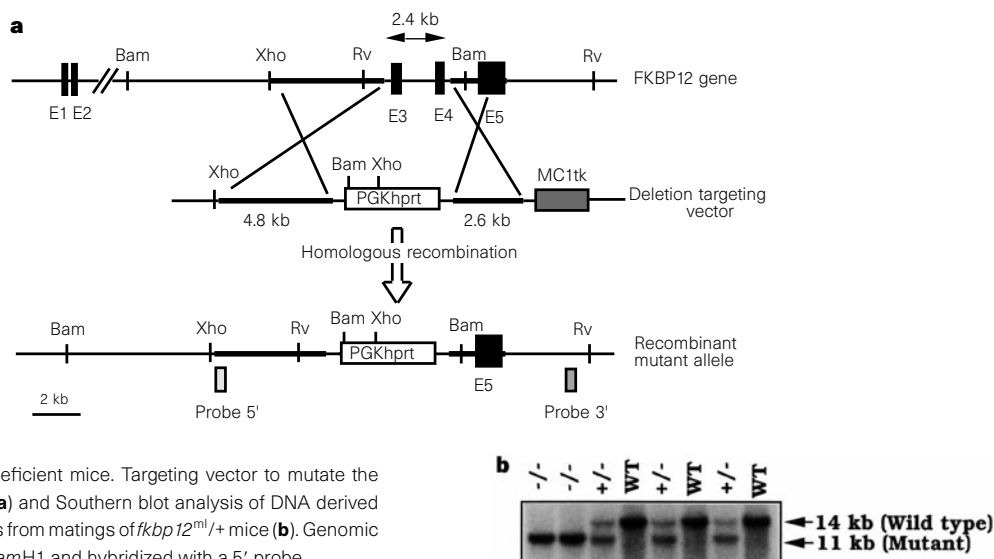


Figure 1 Generation of FKBP12-deficient mice. Targeting vector to mutate the mouse FKBP12 gene in ES cells (a) and Southern blot analysis of DNA derived from a single litter of E18.5 embryos from matings of *fkbp12^{ml}/+* mice (b). Genomic DNA (~5 μ g) was digested with *Bam*H1 and hybridized with a 5' probe.

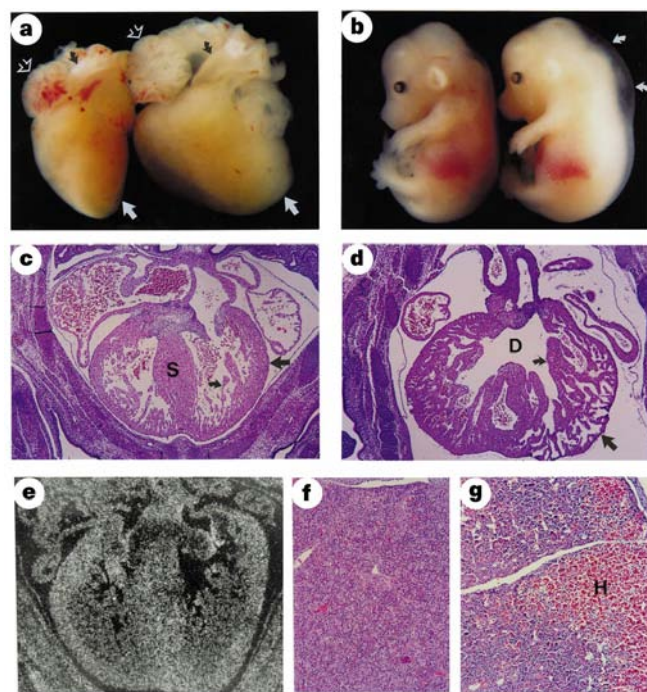


Figure 2 Cardiac and liver analysis of wild-type and FKBP12-deficient mutants. **a**, The E18.5 FKBP12 mutant heart (right) is enlarged compared to the wild-type heart (left). Atria (right atrium, open arrows), ventricles (left ventricle, solid white arrows) and outflow track (small black arrow) are roughly doubled in size in the mutant. **b**, About 50% of E14.5 mutant embryos are oedematous (right, arrows) consistent with an early heart defect^{14,15}. **c**, **d**, Histological analysis of E14.5 FKBP12-deficient (**d**) and littermate control hearts (**c**). The two ventricular walls (large arrow, left ventricle) of the control heart (**c**) are of roughly equal thickness and slightly trabeculated (small arrow). The ventricular septum (S) is prominent. In the FKBP12-deficient heart (**d**), there is a thinned left ventricular wall (large arrow), a prominent ventricular septal defect (D) and hypertrophic trabeculae (small arrow) associated with deep intertrabecular recesses. **e**, *In situ* analysis of FKBP12 mRNA expression in the mouse E13.5 heart. **f**, **g**, Wild-type control (**f**) and FKBP12-deficient (**g**) E14.5 livers. Haemorrhage (H) and necrosis are present in the mutant liver.

(Fig. 3a, b). Cranial neural tube closure is complete by E9.0 in wild-type embryos, but in 9% (6/65) of the FKBP12-deficient E9.5 embryos, neural tube closure defects were present (Fig. 3c, d). Histologically, these mutants demonstrated normal elevation and apposition of the neural folds as well as normal mesenchyme underlying the folds, which is distinct from twist- and cart-1-deficient mice in which disrupted mesenchyme leads to neural tube defects^{16,17}.

As mentioned above, eight FKBP12-deficient mice survived to weaning, and seven of these died within a few weeks because of an apparent cardiac-related wasting syndrome (the eighth lived to 14 months; see below). Both male and female FKBP12-deficient mice had normal sexual differentiation indicating intact Müllerian-inhibiting substance (MIS) and activin signalling pathways¹⁸. These mutants did not have anaemia or pancytopenia or any lymphocyte defects based on complete blood cell analysis and

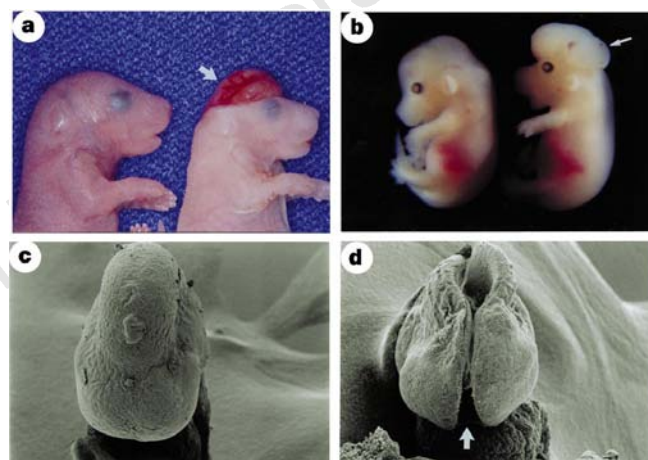


Figure 3 Analysis of wild-type and FKBP12 exencephaly mutants. **a**, 4% of E18.5 FKBP12 mutants have exencephaly (right) in contrast to control littermates (left). **b**, Lateral view of E14.5 wild-type (left) and FKBP12-deficient (right) embryos. Note the 'cauliflower-like' protrusion of the mutant brain (arrow). **c**, **d**, Scanning electron microscopy of wild-type (**c**) and FKBP12-deficient (**d**) E9.5 embryos. The cranial neural tube is completely closed in the wild-type (**c**) but remains open in the mutant (**d**, arrow).

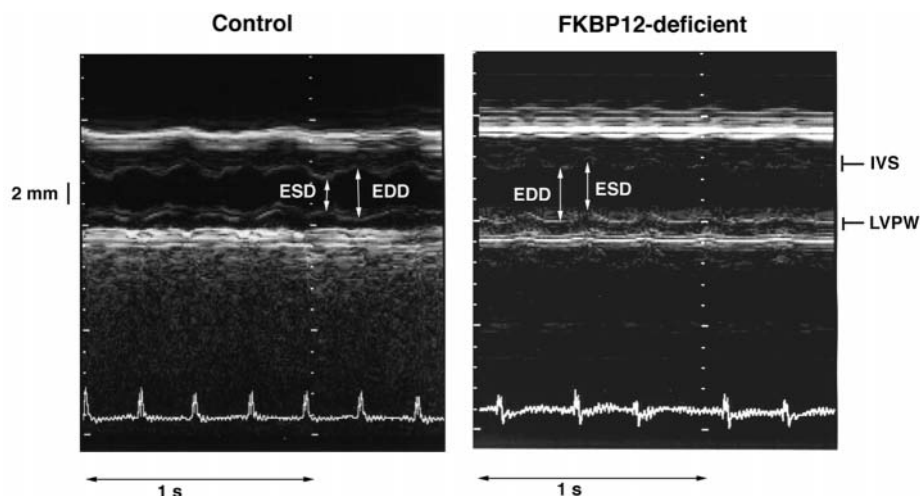


Figure 4 Cardiac performance of adult mice. Transthoracic M mode echocardiographic tracings¹⁹ in a control (left) and an FKBP12-deficient (right) mouse. Vertical arrows indicate left ventricular (LV) chamber at end-diastolic diameter (EDD) and

end-systolic diameter (ESD). Heart rate is indicated at the bottom of each panel. IVS, Intraventricular septum; LVPW, left ventricular posterior wall.

fluorescent-activated cell sorting (FACS) analysis, and the only abnormality was the presence of melanin-containing cells in the spleens of six out of eight mutant mice. Echocardiographic analysis¹⁹ of a 14-month-old FKBP12-deficient male mouse revealed a ventricular septal defect (data not shown) and a dilated left ventricular chamber (that is, end-diastolic diameter (EDD) was greater in the FKBP12-deficient mouse (5.93 ± 0.10 mm) versus the age-matched control (4.93 ± 0.08 mm) mouse ($n = 5$ heart beats; Fig. 4)). The mutant heart showed diminished fractional shortening (%FS = 19.9%) and ejection fraction (%EF = 35.8%) compared to the control heart (%FS = 41.6%; %EF = 65.1%) indicating a depression of contractile activity in the ventricular wall of the mutant heart, similar to MLP-deficient mice²⁰. Thus, the cardiac phenotype of this FKBP12-deficient mouse mimics the clinical features of cardiomyopathy and heart failure in humans.

FKBP12 was postulated to inhibit activin and TGF- β signalling by binding to type I TGF- β /activin receptors^{2,3,7} suggesting that TGF- β -mediated signalling should be enhanced in FKBP12-deficient cells. However, transfection assays with a TGF- β -inducible p3TP-Lux plasmid showed that the induction of luciferase by TGF- β is not significantly different between FKBP12-deficient and control cells (Fig. 5). Thus, the above-mentioned normal sexual differentiation in FKBP12-deficient male and female mice, and these *in vitro* transfection studies demonstrate that the *in vitro* interaction between FKBP12 and activin/TGF- β /MIS type I receptors is not physiologically important. In addition, FKBP12-deficient mice do not phenocopy any of the reported TGF- β superfamily ligand or receptor knockout or overexpressor mouse models¹⁸.

FKBP12 binds and modulates skeletal muscle RyR1^{7,8} and was believed to play a role in skeletal muscle excitation-contraction coupling²¹. However, in contrast to the excitation-contraction uncoupling and muscular degeneration phenotype of RyR1-deficient mice²², FKBP12-deficient mutants failed to demonstrate any gross, histological, or electron microscopic abnormalities in their skeletal muscle (data not shown). To analyse the functional properties of RyR1 function in the absence of FKBP12, skeletal muscle membranes from wild-type and FKBP12-deficient mice were prepared and reconstituted into planar lipid bilayers. RyR1 from FKBP12-deficient mice demonstrated an increased probability of opening compared to RyR1 from wild-type mice, and the channel exists mostly in subconductance states (not fully open) (Fig. 6a, b). RyR1 from wild-type mice remained in a closed state $84 \pm 2\%$ ($n = 3$) of the time whereas RyR1 from FKBP12-deficient mice

remained closed only $16 \pm 4\%$ ($n = 5$) of the time. In addition, the probability of the channel existing at a subconductance state increased over five-fold in the mutants compared to the wild-type mice ($79 \pm 2\%$ versus $13.8 \pm 4\%$, respectively).

The FKBP12 orthologue, FKBP12.6, was believed to bind cardiac RyR2 selectively^{9,10}. However, northern blot analysis of RNA from E18.5 skeletal and cardiac muscle demonstrated low levels of FKBP12.6 messenger RNA which were not upregulated in the mutants (data not shown). To determine whether the cardiac phenotype in FKBP12-deficient mice could be due to defects in cardiac RyR2 function, the single-channel behaviour of RyR2 was examined (Fig. 6c, d). Similar to the RyR1 findings, RyR2 from wild-type mice was closed $80 \pm 7\%$ ($n = 3$) of the time whereas RyR2 from FKBP12-deficient mice was closed $13 \pm 10\%$ ($n = 4$) of the time. Similarly, the amount of time RyR2 was found at a subconductance state increased over six-fold in the FKBP12-deficient mice ($80 \pm 10\%$) versus control mice ($14.4 \pm 7\%$).

Most observations of FKBP12 functions have been based on experiments using an excess of immunosuppressive drugs (such as FK506 and rapamycin) and some of these conclusions have been contradictory. Our lipid bilayer experiments demonstrate that

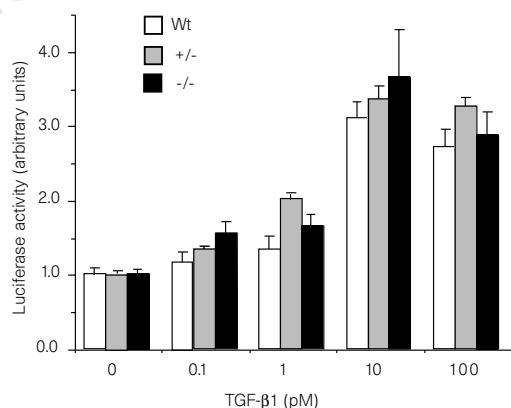


Figure 5 FKBP12 is not essential for TGF- β -mediated signalling. E14.5 fibroblasts isolated from FKBP12-deficient (-/-), heterozygous (+/-) and wild-type (Wt) embryos were transfected with p3TP-Lux and induced by TGF- β at concentrations indicated in the panel as described previously⁴. The relative luciferase activity in the absence of TGF- β was set to one for the wild-type fibroblasts. There was no significant differences in the induction of luciferase (mean $\pm$ s.e., $n = 6$) in the absence of FKBP12.

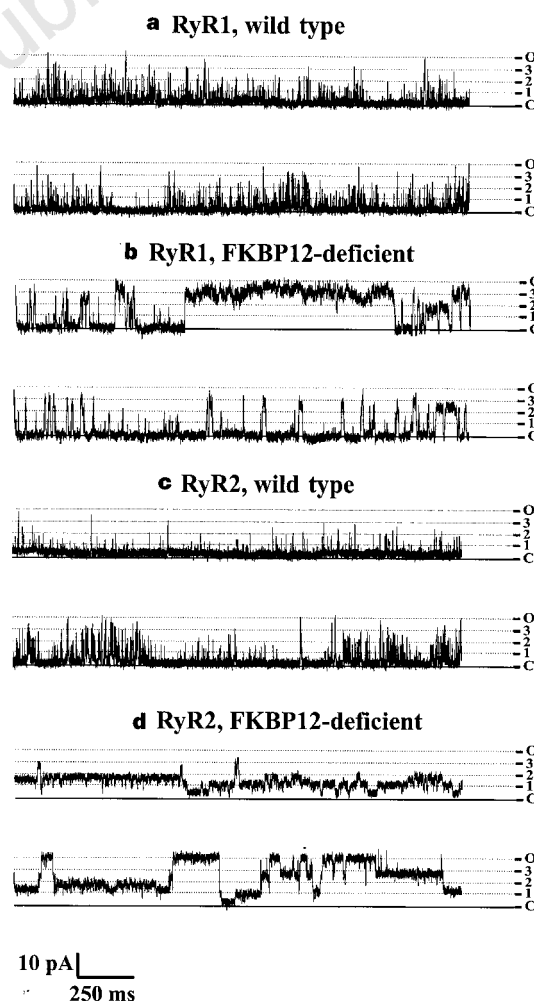


Figure 6 Single channel tracings of skeletal RyR1 (a, b) or cardiac RyR2 (c, d) reconstituted into planar lipid bilayers. The letter 'C' by each tracing refers to the closed state and 'O' refers to the completely open state of the channel. 1, 2, or 3 refer to substates in which the channel is open 1/4, 1/2 or 3/4, respectively. For both the skeletal RyR1 (b) and cardiac RyR2 (d) from FKBP12-deficient mice, the predominant substate is first substate (1/4 amplitude of the completely open channel) with conductance of 120 pS.

absence of FKBP12 alters the single-channel properties of both cardiac RyR2 and skeletal RyR1, that FKBP12.6 cannot functionally replace FKBP12, and that these defects may contribute to the cardiac phenotype in FKBP12-deficient mice. Increased frequency of sub-state channel openings suggests a model whereby FKBP12 is actively involved in the cooperative interactions between subunits of the ryanodine receptor tetramer as presented by Marks and colleagues⁸. Interestingly, the cardiac phenotype of FKBP12-deficient mutants correlates with the cardiac phenotype of humans and chickens treated with the immunosuppressant FK506. It has been reported²³ that five paediatric organ transplant patients, who received high doses of FK506, developed hypertrophic cardiomyopathy associated with clinically severe heart failure that was reversed by switching immunosuppressant therapy from FK506 to cyclosporin. Likewise, it has been reported²⁴ that FK506, but not cyclosporin A, induced cardiac enlargement in chick embryos which subsequently caused embryonic lethality. One enigma from our studies was that the absence of FKBP12 results in abnormal gating properties of both RyR1 and RyR2, yet only cardiac dysfunction is noted. This may reflect differences in excitation-contraction coupling mechanisms between cardiac and skeletal muscle. Cardiac calcium release channels are activated by calcium influx, whereas the skeletal channels are regulated in part by the transverse tubule voltage sensor²⁵. This extra level of control in skeletal muscle might act to prevent opening of the channel in the absence of excitation, thereby decreasing calcium leak from the sarcoplasmic reticulum. Changes in resting cytosolic calcium in cardiac tissue would contribute to the systolic and diastolic dysfunction of FKBP12-deficient embryonic and adult hearts, and subsequently lead to dilated cardiomyopathy and noncompaction of left ventricular myocardium. These FKBP12-deficient mice are an important model for the testing of pharmacological agents and other therapies that may retard or reverse human heart failure. □

Methods

Targeted deletion. FKBP12 genomic clones and four FKBP12 pseudogenes were isolated from a mouse 129SvEv genomic library (Stratagene) using a human FKBP12 complementary DNA. To determine the chromosomal localization of the single-copy mouse FKBP12 gene, we used the Jackson Laboratory backcross DNA panel map service²⁶. The mouse FKBP12 gene was localized to chromosome 2, about 84 cm from the centromere, and cosegregated with a group of 11 loci including *D2Mit22* and *Snta1*, the syntenic region of human chromosome 20 where the human FKBP12 gene maps²⁷. Linearized targeting vector (25 µg) was electroporated into the hprt-negative AB2.1 ES cell line, cell clones were selected in HAT (hypoxanthine, aminopterin and thymidine) and FIAU (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil), DNA from the clones was analysed by Southern blot, and targeted ES cell clone FK35-A12 was expanded and injected into blastocysts as described²⁸. Male chimaeras were bred to C57BL/6J or 129SvEv females to generate F₁ offspring. To confirm that the *fkbp12*^{ml} mutation was null, northern and western blot analyses were performed. No FKBP12 mRNA or protein were observed in homozygous mutants (*fkbp12*^{ml/fkbp12}^{ml}) when compared to wild-type controls (data not shown).

Histological, morphological and *in situ* hybridization analysis. Embryos and tissues were fixed in 10% neutral buffered formalin, paraffin embedded and sectioned (5 µm), and stained with haematoxylin and eosin. For scanning electron microscopy, E9.5 embryos were fixed in Karnovsky's fixative (pH 7.5), rinsed in 0.1 M phosphate buffer (pH 7.3), post-fixed in 1% phosphate buffered osmium tetroxide, dehydrated, and dried to critical point with carbon dioxide. The embryos were then coated with gold-palladium, and examined with a Jeol 6100 scanning electron microscope. *In situ* hybridization was performed as described in ref. 29. Mouse FKBP12 (coding sequence) antisense and sense probes were labelled with [α -³⁵S]UTP using a Promega Riboprobe System. The sense probe did not demonstrate any significant background signal.

Bilayer analysis for single channel activity. Sarcoplasmic reticulum membrane preparations were generated from E18.5 or adult mouse skeletal or cardiac muscle. Experiments were performed as previously described³⁰ and

repeated at least 3 times. Steady-state open probabilities (P_o) were determined by measuring the amount of time that the channel remained open during three minutes of recording. Using [³H] ryanodine, the binding site densities for wild-type skeletal, FKBP12-deficient skeletal, wild-type cardiac, and FKBP12-deficient cardiac tissue were 0.09 ± 0.04 ($n = 3$), 0.10 ± 0.02 ($n = 3$), 0.15 ± 0.05 ($n = 3$) and 0.20 ± 0.04 ($n = 3$) pmol mg⁻¹, respectively.

Isolation of mouse embryonic fibroblasts and transfection assays. Primary fibroblasts were isolated from E14.5 mouse embryos, maintained in DMEM containing 10% FCS, plated at 1×10^5 cells per well in 12-well plates, cultured overnight, transfected with p3TP-Lux and CMV-β-galactosidase reporter plasmids, treated with porcine TGF-β1 (R&D Systems), and analysed for luciferase and β-galactosidase activity 24 h after TGF-β1 treatment as described⁴. Western blot analysis of the wild-type fibroblasts demonstrated significant levels of FKBP12 protein (data not shown).

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Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb

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Members of the Hedgehog (Hh) and Wnt/Wingless (Wg) families of secreted proteins control many aspects of growth and patterning during animal development^{1,2}. Hh signal transduction leads to increased stability of a transcription factor, Cubitus interruptus (Ci)^{3,4}, whereas Wg signal transduction causes increased stability of Armadillo (Arm/ β -catenin)⁵, a possible co-factor for the transcriptional regulator Lef1/TCF⁶. Here we describe a new gene, *slimb* (for supernumerary limbs), which negatively regulates both of these signal transduction pathways. Loss of function of *slimb* results in a cell-autonomous accumulation of high levels of both Ci and Arm, and the ectopic expression of both Hh⁻ and Wg⁻ responsive genes. The *slimb* gene encodes a conserved F-box/WD40-repeat protein related to Cdc4p, a protein in budding yeast

that targets cell-cycle regulators for degradation by the ubiquitin/proteasome pathway⁷⁻⁹. We propose that Slimb protein normally targets Ci and Arm for processing or degradation by the ubiquitin/proteasome pathway, and that Hh and Wg regulate gene expression at least in part by inducing changes in Ci and Arm, which protect them from Slimb-mediated proteolysis.

In a screen for recessive mutations that alter normal adult patterning in *Drosophila*¹⁰, we identified three alleles of a gene we call *slimb* (for supernumerary limbs; abbreviated to *slimb*). One allele, *slimb*¹, behaves like a hypomorph (see Methods): clones of mutant cells generated by mitotic recombination proliferate normally and cause a repertoire of phenotypes characteristic of ectopic activation of the Hedgehog (Hh) signalling pathway (Figs 1, 2). The remaining alleles, *slimb*² and *slimb*^{P1493}, seem to eliminate most or all gene function (Methods): mutant cells undergo only a few divisions and show phenotypes characteristic of ectopic Wingless (Wg) and Hh signal transduction (Fig. 3).

During normal limb development, Hh is expressed by posterior compartment cells and induces adjacent cells in the anterior compartment to express the secreted proteins Decapentaplegic (Dpp) or Wingless (Wg), depending on limb type^{11,12} (Fig. 1e, g). Dpp and Wg then act as morphogens to control growth and patterning in both compartments¹³⁻¹⁶. Clones of *slimb*¹ cells ectopically express *dpp* or *wg* when they arise in the anterior, but not the posterior, compartment (Fig. 1e-h). Further, anterior clones reorganize normal limb pattern, creating supernumerary 'double-anterior' limbs (Fig. 1a, b), whereas posterior clones develop normally. Similar phenotypes are associated with clones of cells which

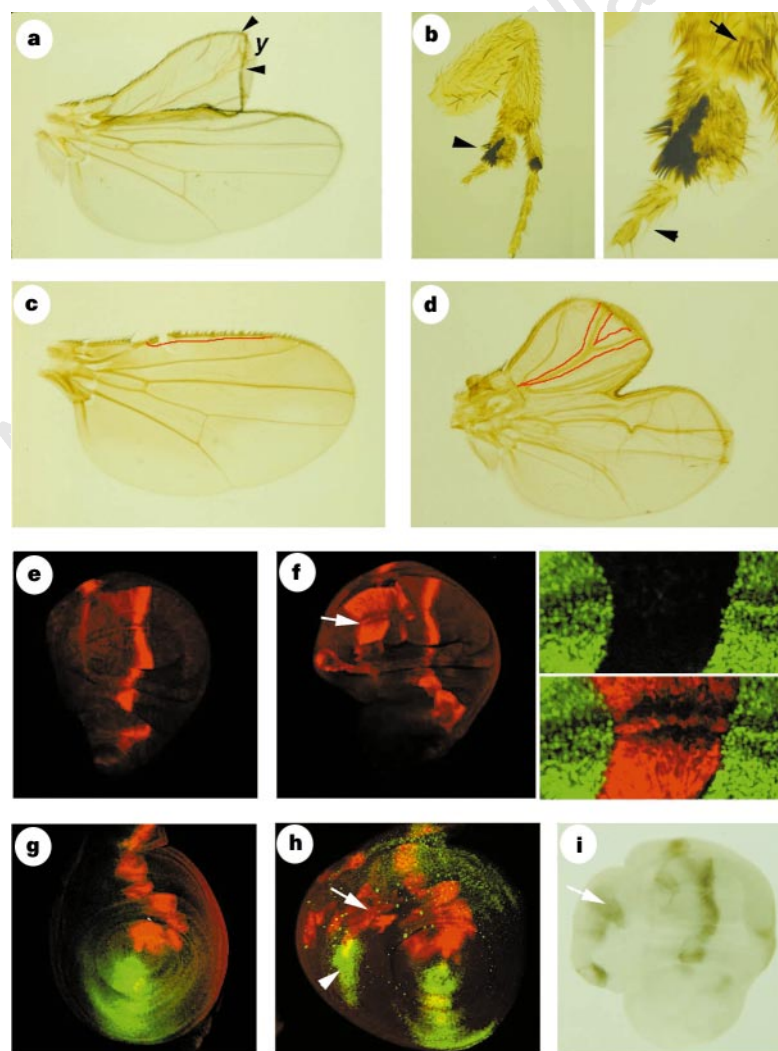


Figure 1 Ectopic activity of the Hh signal transduction pathway in *slimb*¹ mutant cells. **a**, Supernumerary 'double-anterior' wing organized by a clone of homozygous *slimb*¹ cells. Cells belonging to the clone are marked by the yellow (*y*) mutation and form only a small portion of the duplicated appendage (indicated by arrowheads along the wing margin). **b**, Supernumerary male foreleg organized by a clone of *slimb*¹ cells (arrowhead on left panel). The duplicated limb is composed of mirror-symmetric anterior structures such as sex comb teeth and characteristic dorsal and ventral bristles (arrow and arrowhead, respectively, on right panel). **c**, A clone of *dpp*^{d12}/*dpp*^{d12}; *slimb*¹/*slimb*² mutant cells. Cells belonging to the clone are marked by the *stc* mutation¹⁰, and the border of the clone along the dorsal wing surface is shown in red. **d**, Supernumerary wing organized by a clone of *smo*³/*smo*³; *slimb*¹/*slimb*² mutant cells (marked as in **c**). **e**, A wild-type wing disc showing the normal expression of a *dpp-lacZ* reporter gene (red) in a stripe of cells just anterior to the anterior-posterior compartment boundary in response to endogenous Hh. **f**, A wing disc carrying *slimb*¹ mutant clones. Cells belonging to the clone indicated by the arrow are marked by the absence of expression of a Myc-tagged form of GFP (green). Note that the *dpp-lacZ* gene is autonomously expressed by the mutant cells but not by neighbouring *slimb*¹ cells. **g**, A wild-type leg disc showing the normal expression of a *dpp-lacZ* reporter gene (red) dorsally and Wg (green) ventrally induced by Hh in cells just anterior to the anterior-posterior compartment boundary. **h**, A leg imaginal disc carrying several *slimb*¹ clones. Dorsally situated *slimb*¹ cells in the anterior compartment (arrow) ectopically express the *dpp-lacZ* reporter gene (red), whereas ventrally situated *slimb*¹ cells (arrowhead) ectopically express Wg (green). **i**, A wing disc carrying clones of *smo*³/*smo*³; *slimb*¹/*slimb*² mutant cells and stained for Dpp expression. The arrow marks ectopic Dpp expression, which we infer is associated with a clone of mutant cells. The ectopic Dpp-expressing cells have organized a presumptive secondary appendage which appears as an abnormal outgrowth of the disc epithelium.

ectopically express Hh¹¹, or in which the Hh signal transduction pathway is constitutively active owing to the loss of activity of protein kinase A (PKA) (reviewed in ref. 17). However, *slmb*¹ cells ectopically express *dpp* and *wg* in a strictly cell-autonomous manner (Fig. 1f and data not shown), in contrast to ectopic Hh-secreting cells which induce *dpp* and *wg* gene activity in neighbouring cells¹⁸. Moreover, the reorganizing activity of *slmb*¹ cells can be attributed specifically to this ectopic *dpp* and *wg* expression as it depends on *dpp* and *wg* gene activity in the same cells (Fig. 1c and data not shown). Finally, clones of *slmb*¹ cells that lack the function of Smoothed (Smo), a transmembrane protein essential for Hh signal reception¹⁹, still ectopically express Hh-responsive genes such as *dpp* (Fig. 1i) and cause the formation of supernumerary limbs (Fig. 1d). We conclude that Slimb, like PKA, is a negative

regulator that normally prevents activity of the Hh signal transduction pathway in the absence of ligand.

Hh signalling controls gene expression by regulating the level and state of Cubitus interruptus (Ci), a transcription factor which activates the expression of Hh-responsive genes (reviewed in ref. 20). Ci is expressed only in anterior compartment cells and is truncated at the carboxy-terminal end by proteolysis in the absence of Hh signalling⁴. However, this proteolytic processing is inhibited in cells exposed to Hh, leading to the accumulation of high levels of intact Ci, which can be detected by a monoclonal antibody that recognizes an epitope in the C terminus^{3,4} (Fig. 2a). We find that *slmb*¹ mutant cells in the anterior compartment autonomously accumulate high levels of Ci protein carrying this epitope (Fig. 2a, b, d, e), although transcription of the *ci* gene remains unchanged (Fig. 2c). By using an antibody that recognizes an amino-terminal epitope of Ci, we observe that Ci is found predominantly in the full-length form in *slmb*¹/*slmb*² mutant cells (Fig. 2f), as it is in PKA mutant cells (Fig. 2f) and cells exposed to ectopic Hh⁴, rather than in the truncated form found in the absence of Hh signalling⁴. These results indicate that Ci is proteolytically processed in the absence of Hh signalling as a consequence of Slimb and PKA activity, and suggest that Hh alleviates this negative regulation, leading to an increase in the level of full-length Ci.

Like *slmb*¹ cells, *slmb*^{P1493} cells accumulate high levels of intact Ci (data not shown), indicating that the Hh transduction pathway is constitutively active in the mutant cells. However, they also show

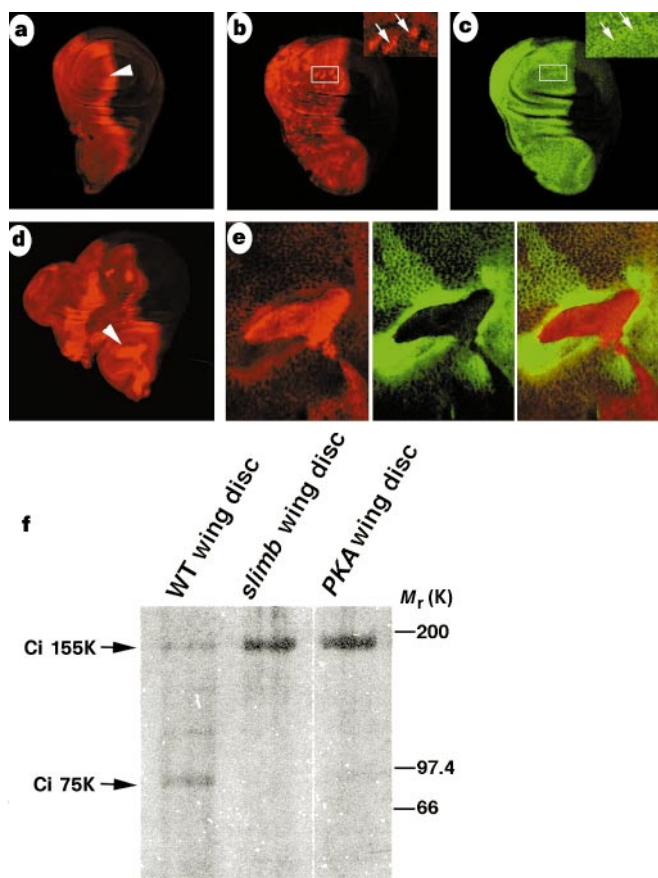


Figure 2 Accumulation of intact Ci protein in *slmb*¹ mutant cells. **a**, A wild-type wing disc stained to show the accumulation of high levels of intact Ci protein (red) along the anterior-posterior compartment boundary (arrowhead), as detected by a monoclonal antibody against the carboxy-terminal portion of the protein (mAb 2A1)³. **b**, **c**, Wing disc carrying several *slmb*¹ clones and stained to show expression of intact Ci (**b**, red) and expression of a *ci-lacZ* reporter gene (**c**, green). The islands of cells expressing high levels of intact Ci correspond to *slmb*¹ clones (see **d**). The *ci-lacZ* gene appears to be expressed at the same level in mutant and wild-type cells. **d**, **e**, Wing disc carrying several clones of *slmb*¹ cells marked by the loss of expression of the CD2 reporter protein (green); the arrow marks a clone of *slmb*¹ mutant cells which can be seen to accumulate high levels of intact Ci (red) in a cell-autonomous manner in **e**. **f**, Western blot analysis of Ci derived from wild-type (WT) wing discs, *slmb*¹/*slmb*² wing discs, and wing discs containing a high frequency of *PKA*⁻ cells (generated using the Minute technique²⁰). An antiserum directed against the aminoterminal of Ci (AbN)⁴ shows the predominant accumulation of intact Ci in *slmb* mutant wing discs and *PKA* mutant clone-bearing wing discs, in contrast to wild-type discs in which most of the Ci corresponds to the processed C-terminally truncated form (Ci 75K)⁴.

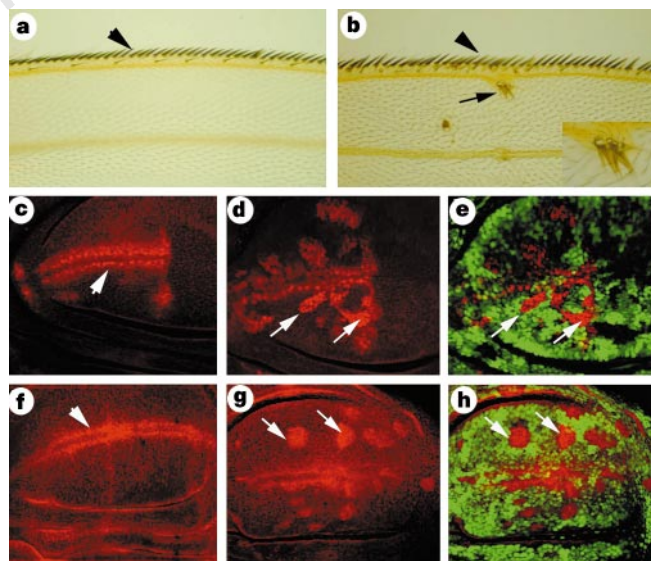


Figure 3 Ectopic activity of the Wg signal transduction pathway in *slmb*^{P1493} mutant cells. **a**, **b**, Dorsal surface of a wild-type wing (**a**) and a wing carrying *slmb*^{P1493} clones (**b**). The sensory bristles formed along the wing margin are indicated by an arrowhead in each panel; *slmb*^{P1493} cells are marked with the *y* mutation and differentiate autonomously as ectopic wing-margin bristles (arrow in **b**; insert). **c**–**e**, Scute (Sc) expression (red) in a wild-type wing disc (**c**) and in a wing disc carrying *slmb*^{P1493} mutant clones (**d**, **e**). At this stage, Sc is expressed as two stripes (arrowhead) flanking the presumptive wing margin; *slmb*^{P1493} cells (arrows; marked as in Fig. 1f by the loss of expression of Myc-tagged GFP) ectopically express Sc in a cell-autonomous manner. **f**–**h**, Arm expression (red) in a wild-type wing disc (**f**) and a wing disc carrying *slmb*^{P1493} clones (**g**, **h**). In the wild-type disc, Arm is expressed at low levels in the wing porch, except in the presumptive wing margin where it is accumulated to high levels in two stripes straddling the dorsoventral compartment boundary (arrowhead); *slmb*^{P1493} cells (marked by the lack of green Myc-GFP expression) autonomously accumulate high levels of Arm protein (arrows).

additional phenotypes not associated with *smb*¹ cells, and these phenotypes indicate that the Wg signal transduction pathway is also constitutively activated.

During wing development, *wg* expression is normally induced by Delta and Serrate signalling in a thin strip of cells along the dorsoventral compartment boundary (reviewed in ref. 21). Wg protein emanating from these cells then induces the expression of Scute (Sc), a proneural gene product that specifies the formation of sensory bristles on the wing margin (Fig. 3a; reviewed in ref. 22), and acts as a morphogen to control the development of the wing blade^{15,16}. We found that *smb*^{P1493} cells which arise in the presumptive wing blade ectopically express Sc (Fig. 3c–e) and differentiate ectopic sensory bristles instead of epidermal hairs on the surface of the wing blade (Fig. 3b). Both phenotypes are strictly autonomous to the mutant cells (Fig. 3b, e), as is the case when the Wg signal transduction pathway is constitutively activated^{15,16}, but not when Wg is ectopically expressed^{15,16}.

Wg signal transduction is associated with the accumulation of high levels of Arm (reviewed in ref. 5), as is evident in the developing wing blade where it accumulates to higher levels in association with Wg-secreting cells along the presumptive wing margin (Fig. 3f). As shown in Fig. 3g, h, *smb*^{P1493} mutant cells autonomously express high levels of Arm protein wherever they arise in the presumptive wing blade (Fig. 3g, h). Hence Slimb seems to be involved in

destabilizing Arm in the absence of Wg signalling, just as it down-regulates the accumulation of full-length Ci in the absence of Hh signalling.

We cloned *smb* by P-element tagging and identified a coding sequence that can completely rescue each of the three mutant alleles of *smb* when expressed under the constitutive control of the *Tubulin*α1 promoter (Methods; Fig. 4). Conceptual translation of the open reading frame (ORF) indicates that *smb* encodes a member of the F-box/WD40-repeat family of protein which includes the yeast cell-cycle proteins Cdc4 (ref. 7) and Fop1 (ref. 23). In budding yeast, Cdc4p acts in conjunction with Skp1p, Cdc53p and Cdc34p (a ubiquitin-conjugating enzyme) to promote ubiquitination and proteolysis of cell-cycle regulators such as the CDK inhibitor Sic1p (refs 7–9). Cdc4p interacts with phosphorylated Sic1p and recruits it to the ubiquitin–ligase complex composed of Cdc4p, Skp1p and Cdc53p (refs 8, 9). Thus Slimb may play an analogous role to Cdc4p, targeting Ci and Arm for ubiquitination and proteolysis by the proteasome. In the case of Ci, interactions with Slimb would lead to a selective processing event mediated by the ubiquitin/proteasome pathway, perhaps similar to that which occurs to the NF-κB precursor protein²⁴. In the case of Arm, such interactions would result in degradation. Accordingly, we suggest that Hh and Wg alter the state of Ci and Arm, respectively, allowing these proteins to escape from

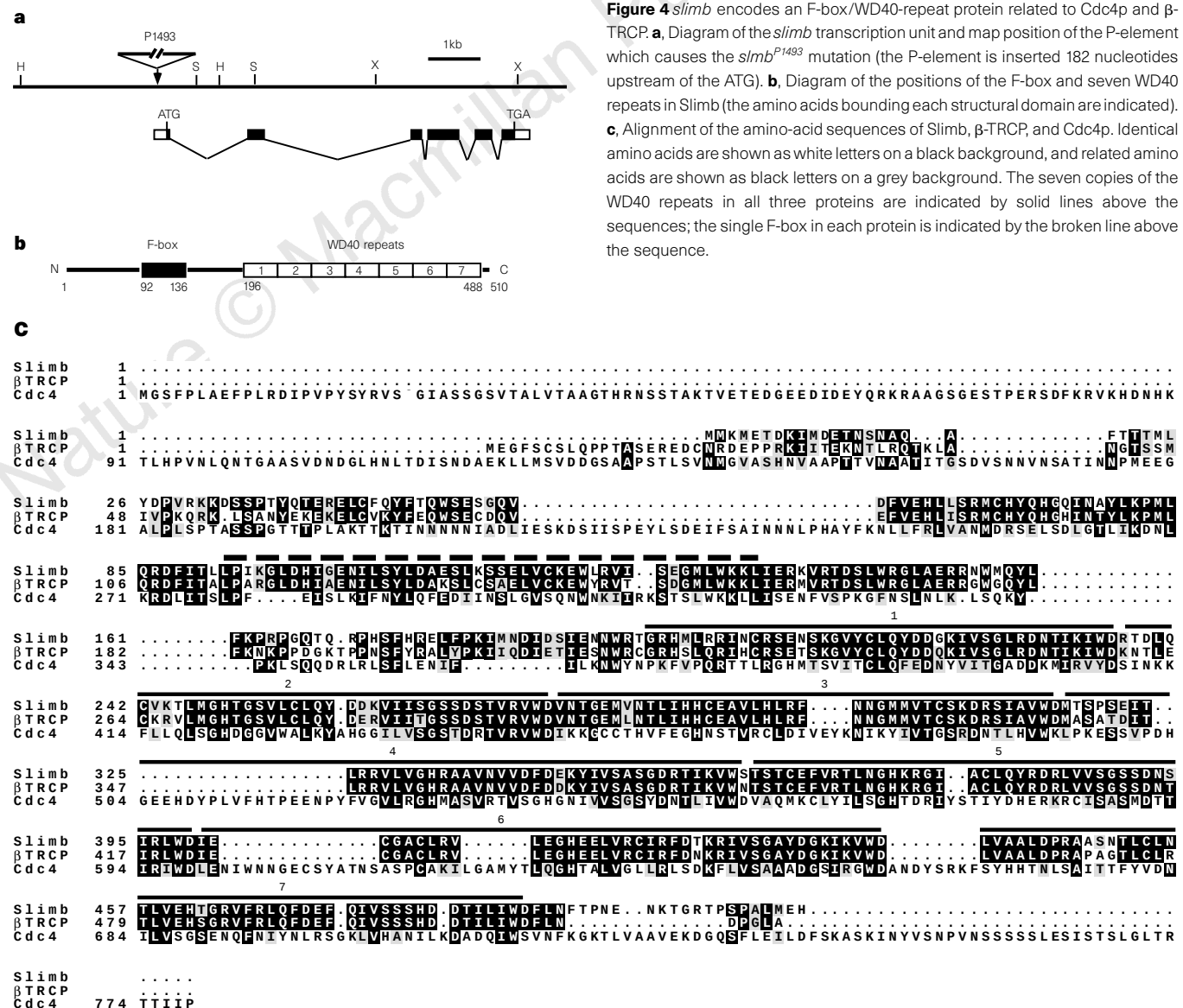


Figure 4 *smb* encodes an F-box/WD40-repeat protein related to Cdc4p and β -TRCP. **a**, Diagram of the *smb* transcription unit and map position of the P-element which causes the *smb*^{P1493} mutation (the P-element is inserted 182 nucleotides upstream of the ATG). **b**, Diagram of the positions of the F-box and seven WD40 repeats in Slimb (the amino acids bounding each structural domain are indicated). **c**, Alignment of the amino-acid sequences of Slimb, β -TRCP, and Cdc4p. Identical amino acids are shown as white letters on a black background, and related amino acids are shown as black letters on a grey background. The seven copies of the WD40 repeats in all three proteins are indicated by solid lines above the sequences; the single F-box in each protein is indicated by the broken line above the sequence.

Slimb-mediated proteolysis and accumulate to high levels. For example, Ci and Arm may normally be phosphorylated by PKA and glycogen synthase kinase 3 (GSK3; reviewed in refs 5, 17), and signalling by Hh and Arm may change the state or degree of their phosphorylation, blocking their ability to interact with Slimb.

Slimb has ~80% amino-acid identity to β -TRCP, a *Xenopus* protein of unknown function²⁵. In *Xenopus* and mammalian cells, β -catenin, a homologue of *Drosophila* Arm, seems to be targeted for degradation by the ubiquitin/proteasome pathway in the absence of Wg/Wnt signalling^{26,27}. Hence, the close structural homology between β -TRCP and Slimb raises the possibility that the two proteins have a conserved role in destabilizing Arm/ β -catenin. Mutations in other genes which allow β -catenin to escape from degradation are associated with a wide range of metastatic disorders²⁸. We therefore speculate that mammalian homologues of *slimb* may be tumour-suppressor genes. □

Methods

Mutations and transgenes. *slimb*¹ and *slimb*² are EMS-induced mutations isolated from a screen for mutations that cause abnormal adult patterns in somatic clones¹⁰. *slimb*¹ and *slimb*² are lethal *in trans* to each other and *in trans* to deficiency *Df(3R)3-R1*, which eliminates the chromosome interval 93B3-5 to 93D2-4. *slimb*¹/*slimb*² and *slimb*¹/*Df(3R)3-R1* animals survive to the pupal stage; imaginal discs isolated from *slimb*¹/*slimb*² larvae show ectopic Dpp and Wg expression as well as high levels of Ci and excessive proliferation in anterior compartment cells. *slimb*²/*Df(3R)3-R1* animals die during early larval life. *slimb*^{P1493} is a P-element insertion mutation at cytological position 93B10-11, originally called *l(3)00295*. *slimb*^{P1493} behaves like *Df(3R)3-R1* in complementation tests with *slimb*¹ and *slimb*², resulting in pupal and early larval lethality, respectively. Wild-type function can be restored by precise P-element excision. The *smo*³, *dpp*^{d12} and *PKA*⁻ mutations, as well as the *dpp-lacZ3.0* and *ci-lacZ* reporter genes, have been described^{10,11,29}. *Tuba1-slimb* is a transgene containing the entire *slimb* ORF under the control of the constitutive *Tubulin1* promoter¹¹ inserted on the left arm of the second chromosome (see below). *hsp70-Myc-tagged GFP* is a transgene containing the coding sequence for a Myc-tagged form of green fluorescent protein (GFP) under the control of the heat-shock inducible promoter of the *hsp70* gene (A. Adachi and G. Struhl, unpublished).

Cloning of *slimb*. The genomic DNA sequence flanking the insertion site of *slimb*^{P1493} was isolated by plasmid rescue and used as a probe to screen an ovarian cDNA library. A full-length sequence for the *slimb* ORF was assembled from several partial cDNA clones. The identity of the *slimb* ORF is verified by rescue experiments: *slimb*² or *slimb*^{P1493} homozygotes as well as *slimb*¹/*slimb*² heterozygotes that carry a single copy of *Tuba1-slimb* transgene are viable and show normal morphology, except that 5–10% of the adults exhibit duplications of the haltere.

Generating clones of marked mutant cells. Clones of mutant cells were generated by FLP/FRT-mediated mitotic recombination as described¹⁰. Genotypes for generating clones are as follows. *slimb* mutant clones: *y hsp-flp.1/y* or *Y; FRT82E slimb^{1,2} or P1493/FRT82E hsp-CD2, y⁺ (or hsp70-myc-gfp, w⁺)* with or without *dpp-lacZ3.0* on the second chromosome. *slimb dpp* clones: *y hsp-flp.1/y* or *Y; dpp^{d12} stc FRT 39E/Tuba1-slimb FRT39E; slimb¹/*slimb*². *slimb smo* mutant clones: *y hsp-flp.1/y* or *Y; smo³ stc FRT 39E/Tuba1-slimb FRT39E; slimb¹/*slimb*².**

Imaginal disc staining and western blot analysis. Standard protocols for immunofluorescence and immunohistochemistry of imaginal discs were used¹⁰. Western blot analysis was performed as described⁴.

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Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c

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Following exposure of cells to stimuli that trigger programmed cell death (apoptosis), cytochrome c is rapidly released from mitochondria into the cytoplasm where it activates proteolytic molecules known as caspases that specifically cleave the amino-acid sequence DEVD and are crucial for the execution of apoptosis^{1–4}. The protein Bcl-2 interferes with this activation of caspases by preventing the release of cytochrome c^{2–4}. Here we study these molecular interactions during apoptosis induced by the protein Bax, a pro-apoptotic homologue of Bcl-2 (refs 5, 6). We show that

in cells transiently transfected with *bax*, Bax localizes to mitochondria and induces the release of cytochrome *c*, activation of caspase-3, membrane blebbing, nuclear fragmentation, and cell death. Caspase inhibitors do not affect Bax-induced cytochrome *c* release but block caspase-3 activation and nuclear fragmentation. Unexpectedly, Bcl-2 also fails to prevent Bax-induced cytochrome *c* release, although it co-localizes with Bax to mitochondria. Cells overexpressing both Bcl-2 and Bax show no signs of caspase activation and survive with significant amounts of cytochrome *c*

in the cytoplasm. These findings indicate that Bcl-2 can interfere with Bax killing downstream of and independently of cytochrome *c* release.

Rat (R6) embryo fibroblasts and human SK2 melanoma transfected with *bax* were monitored for Bax expression and cytochrome *c* release by immunocytochemistry and for nuclear condensation/fragmentation by Hoechst staining. Immunostaining of endogenous rat Bax was filamentous (Fig. 1a), whereas that of transfected human Bax was punctuated (Fig. 1c, e). However, both forms of Bax

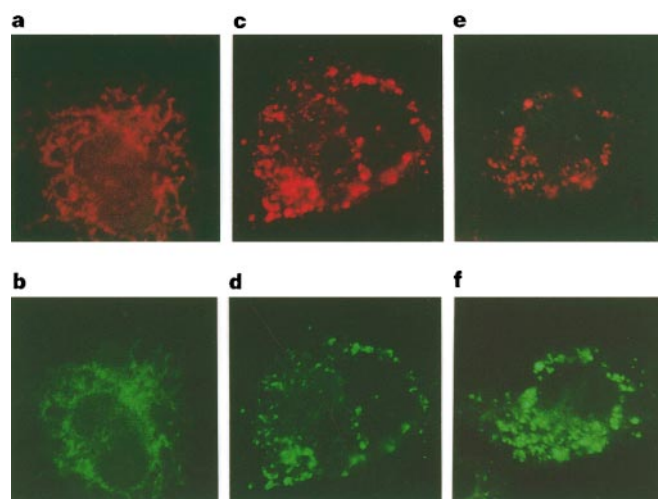


Figure 1 Bax co-localizes with mitochondrial cytochrome *c* oxidase VIc (COX) and Bcl-2. Anti-Bax (a, c, e), anti-COX (b, d) and anti-Bcl-2 (f) immunocytochemical analysis on non-transfected (a, b), *bax*-transfected (c, d) and *bax/bcl-2*-transfected (e, f) R6 cells at 8 h post-transfection. Magnification, $\times 1,000$.

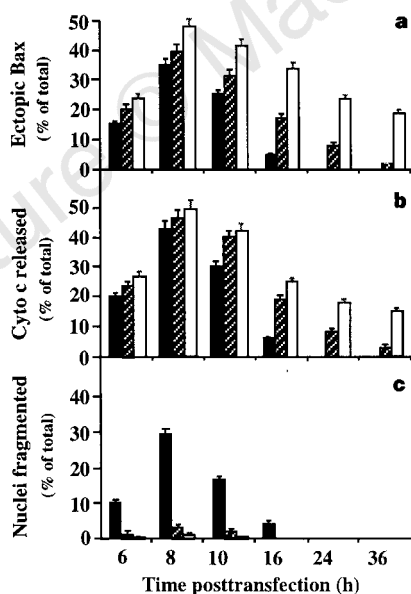


Figure 2 Quantification of ectopic Bax expression, cytosolic cytochrome *c* and fragmented nuclei following *bax* transfection into R6 and SK2 cells. The cells were transfected with *bax* (black bars) in the absence or presence of Z-DEVD-fmk (hatched bars) or co-transfected with *bax* and *bcl-2* (white bars) and subjected to anti-Bax, anti-cytochrome *c* and Hoechst 33342 analysis at various times post-transfection as for Fig. 3. The percentages of cells displaying punctuated Bax (a), diffuse cytochrome *c* (b) or fragmented nuclei (c) was determined by counting fluorescent cells on 20 randomly selected fields under the microscope. 100% represents total cells of the respective fluorescence per field. Data are depicted as means $\pm$ s.d. from two independent transfections into R6 and SK2 cells, respectively (total of 80 fields analysed).

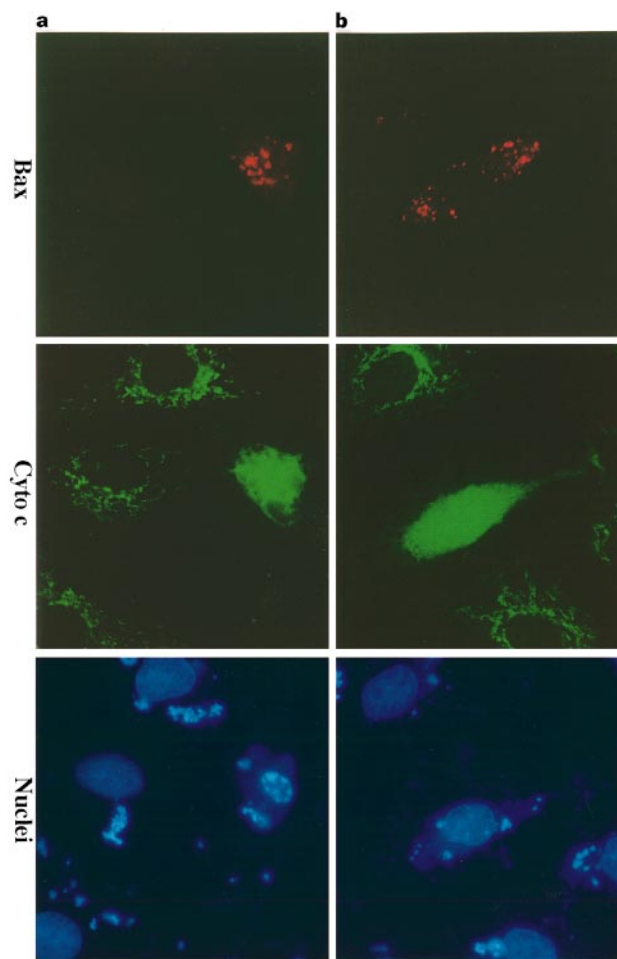


Figure 3 Bax induces cytochrome *c* release irrespective of Bcl-2 inhibition. Anti-Bax (rhodamine), anti-cytochrome *c* (fluorescein) and Hoechst 33342 (UV) fluorescence analysis on *bax*-transfected (a) and *bax/bcl-2*-transfected (b) R6 cells at 8 h post-transfection. Hoechst-stained aggregates around the nuclei are DNA/Superfect complexes that could not be entirely removed after transfection. Magnification, $\times 1,000$.

co-localized with the mitochondrial cytochrome *c* oxidase subunit VIc (COX) (Fig. 1b, d), indicating that ectopic Bax changed the mitochondrial morphology from a filamentous to a punctuated pattern. This change was specific for Bax overexpression as it occurred in only 0.1% of non-transfected cells (data not shown). The strong anti-Bax immunostaining allowed cells overexpressing Bax to be counted by fluorescence microscopy. At 6 h post-transfection, about 15% of total cells were positive for ectopic Bax (Fig. 2a). This number increased to 35% at 8 h post-transfection but decreased thereafter because of cell loss by apoptosis (Fig. 2a).

We next studied the fate of cytochrome *c* in *bax*-transfected R6 and SK2 cells. The immunostaining of cytochrome *c* was filamentous in non-transfectants but was diffuse in cells expressing punctuated Bax, suggesting that ectopic Bax may have provoked a redistribution of cytochrome *c* from the mitochondria to the cytoplasm (Fig. 3a). Cells displaying diffuse cytochrome *c* staining were all apoptotic because their nuclei were condensed and/or fragmented (Figs 2b, c and 3a). To study the role of caspases in this type of cell death, we treated *bax* transfectants with cell-permeable caspase inhibitors, Z-DEVD-fmk (Fig. 2) or Z-VAD-fmk (data not shown). As shown in Fig. 2, such treatments prolonged the survival of cells overexpressing Bax after release of cytochrome *c*. The nuclei of these cells were not fragmented but remained condensed, and all cells were detached from the coverslip at 36 h post-transfection (Fig. 2). These results indicate that caspases act downstream of cytochrome *c* to trigger Bax-induced nuclear fragmentation, but that there is a pathway to cell death that cannot be blocked by caspase inhibitors.

To confirm our results, we transfected human *bax* into HEK 293T cells. Owing to the very high efficiency of transfection, these cells allowed a biochemical analysis of caspase-3 activation and DNA fragmentation. Transfectants were identified by X-Gal blue staining following co-transfection with *bax* and β -galactosidase (β -gal) as previously described⁷. Apoptotic cells were round and blebbing, whereas surviving cells were elongated in shape (Fig. 4a). Between 6 and 10 h post-transfection, elongated blue cells (Fig. 4b, white bars) showed increased blebbing (Fig. 4b, black bars) and were shedded into the medium. Thus, only a few blue cells remained attached to the plate by 16 h post-transfection (Fig. 4b). Western blot analysis revealed that between 6 and 10 h post-transfection, 20–40% of total cytochrome *c* was redistributed from a membrane to a soluble fraction (Fig. 4c). This coincided with the conversion of the inactive 32K pro-caspase-3 into the active 17K protease (Fig. 4d) and was followed by the appearance of nucleosome-sized DNA fragments at 8–16 h post-transfection (Fig. 4e). At 16–24 h post-transfection, cytosolic cytochrome *c*, p17 caspase-3, and DNA fragments had all completely disappeared, presumably because apoptotic cells were lost (Fig. 4c–e). Again, the caspase inhibitors Z-DEVD-fmk (Fig. 4c–e) and Z-VAD-fmk (data not shown) did not inhibit cytochrome *c* release but blocked both caspase-3 activation and DNA fragmentation. Thus, a large number of blue cells still adhered to the plate (Fig. 4b) and expressed cytosolic levels of cytochrome *c* at 16 h post-transfection (Fig. 4c). Most of these cells displayed a blebbing phenotype (Fig. 4b, filled bars), however, and shedded off the plate later on, confirming that caspase inhibitors can delay but not prevent Bax-induced cytotoxicity.

Bcl-2 interacts with Bax and blocks Bax-induced apoptosis^{5,6}. We therefore co-transfected equal amounts of *bcl-2* and *bax* into R6 and SK2 cells and studied the effect of Bcl-2 on Bax-induced cytochrome *c* release, caspase activation and nuclear fragmentation. As previously seen with Bax and mitochondrial COX (Fig. 1c, d), Bcl-2 and Bax co-immunolocalized in a punctuated pattern (Fig. 1e, f), indicating that overexpressed Bcl-2 encountered Bax on the mitochondria. Consistent with this, Bcl-2 and Bax co-immunoprecipitated from radiolabelled extracts of cells that stably overexpressed Bcl-2 and Bax⁸. At any time post-transfection, Bcl-2 coexpression increased the number of cells positive for punctuated, ectopic Bax

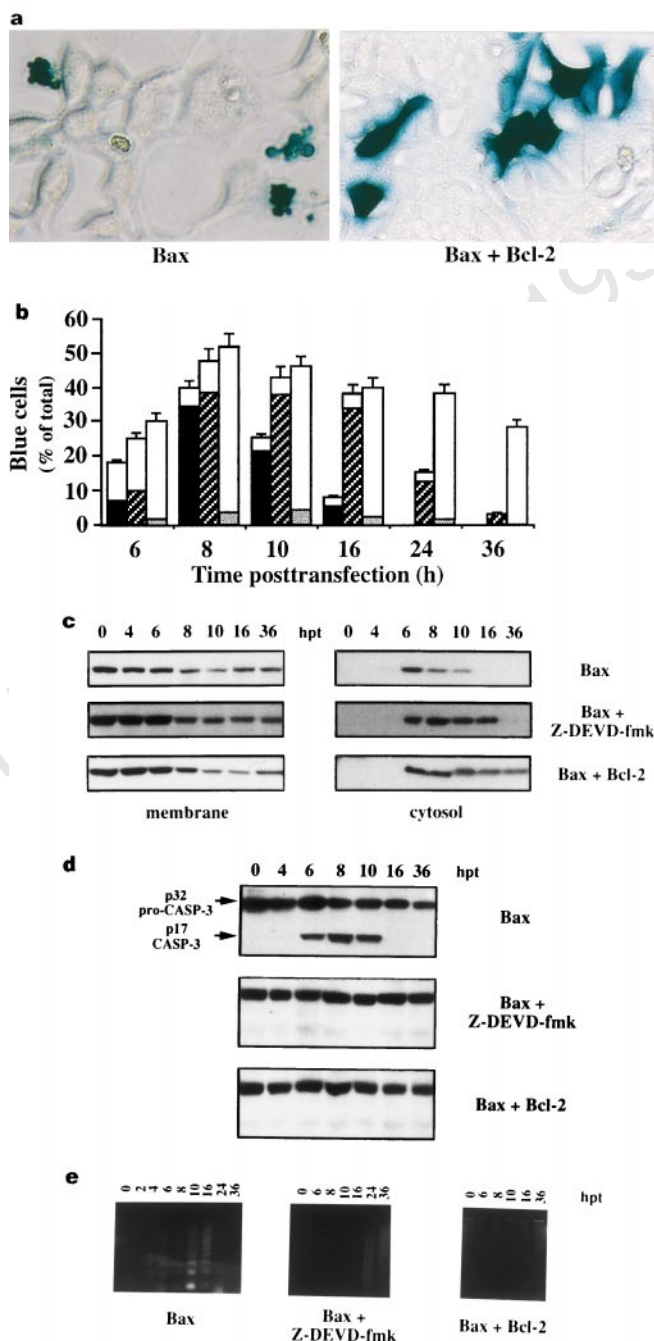


Figure 4 Apoptotic morphology, cytochrome *c* release, caspase-3 activation and DNA fragmentation in response to Bax or Bax/Bcl-2 expression in HEK 293T cells. At various times after transfection with *bax*/ β -gal or *bax*/*bcl-2*/ β -gal in the presence or absence of Z-DEVD-fmk, 293T cells were stained with X-Gal and counted, or disrupted and analysed for cytochrome *c* expression, caspase-3 activation and DNA fragmentation as described in Methods. **a**, Blue-stained, *bax*-transfected 293T cells are round and blebbing (left), whereas the respective *bax*/*bcl-2*-transfectants show an intact, elongated morphology (right). Pictures were taken at 10 h post-transfection. **b**, Quantification of blue cells with blebbing and with normal morphology. The percentage of blebbing blue cells per plate (out of total cells) are represented by filled bars (black for *bax* transfectants, hatched for *bax* transfectants with Z-DEVD-fmk, shaded for *bax*/*bcl-2* co-transfectants); results for the respective normal blue cells are represented by white bars. The data are means $\pm$ s.d. from 20 field countings of three independent transfections. **c**, Anti-cytochrome *c* immunoblot of cytosolic and membrane fractions. **d**, Anti-caspase-3 immunoblot of total protein. The positions of the inactive 32K pro-form and the active 17K form are indicated by arrows. **e**, DNA fragmentation analysis. hpt, Hours post-transfection.

(Fig. 2a), and the nuclei of these cells were not fragmented (Figs 2c, 3b). Some of the *bax/bcl-2* co-transfectants even survived for 36 h (Fig. 2a). Unexpectedly, however, Bcl-2 was incapable of blocking Bax-induced cytochrome *c* release because all cells overexpressing Bax displayed a diffuse immunostaining of cytochrome *c* (Figs 2b, 3b). Western blot analysis of extracts from HEK 293T cells co-transfected with *bcl-2* and *bax* confirmed that significant levels of cytochrome *c* remained in the cytoplasm for up to 36 h (Fig. 4c). Both caspase-3 activation and DNA fragmentation were blocked and only a few blebbing cells were detected after X-Gal staining of *bcl-2/bax/β-gal* triple transfectants at any time post-transfection (Fig. 4a–e). Even at 36 h post-transfection, we still noted blue adherent 293T cells with an intact, elongated morphology (Fig. 4b). Thus, Bcl-2 can act after cytochrome *c* release to prevent Bax-induced caspase-3 activation, nuclear fragmentation and cytotoxicity.

We have shown that Bax triggers the same sequence of cellular events as previously described for other apoptotic agents^{1–4}: the release of cytochrome *c* from mitochondria into the cytoplasm, followed by the activation of death-effector caspases, nuclear fragmentation and apoptosis. However, two features are different: (1) Bax provokes a pathway to cell death not blocked by the caspase inhibitors Z-DEVD-fmk and Z-VAD-fmk^{9,10}, and (2) Bcl-2 seems to have a caspase-inhibiting activity downstream or aside from cytochrome *c* release. The latter may be due to an interaction of Bcl-2 with the cytochrome *c* receptor Apaf-1/CED-4 (ref. 11), a recently identified mediator of caspase-3 activation by cytochrome *c*. □

Methods

Transient transfection into R6 and SK2 cells and immunofluorescence analysis. 5×10^4 rat R6 embryo fibroblasts or SK2 human melanoma cells grown on glass coverslips were transfected with either 1 μ g of each Bax/pcDNA3 and pcDNA3 or Bax/pcDNA3 and Bcl-2/pcDNA3 using 10 μ g of Superfect (Qiagen) as described by the manufacturer. In some experiments, 50 μ M of the caspase inhibitors Z-VAD-fmk or Z-DEVD-fmk (Bachem) were added at the beginning and at every 12 h post-transfection. At various times post-transfection, cells were fixed and permeabilized in 4% paraformaldehyde/0.05% saponin containing 4 μ g ml⁻¹ Hoechst 33342 dye (Molecular Probes) to visualize nuclei and DNA. Cells were then treated with hamster monoclonal anti-Bcl-2 3F11 (Pharmingen), rabbit polyclonal anti-Bax 06-499 (Upstate Biotechnology), mouse monoclonal anti-cytochrome *c* 6H2.B4 (Pharmingen) or mouse monoclonal anti-cytochrome *c* oxidase subunit VIc (COX) (Molecular Probes) antibodies, and then incubated with rhodamine- and fluorescein-conjugated secondary antibodies (Jackson Laboratories). The antifading agent SlowFade (Molecular Probes) was added and cells were viewed under a Zeiss Axiovert fluorescence microscope. Fluorescent cells were counted on 20 randomly selected fields (40–50 cells per field) under the microscope and the number of cells positive for punctuated Bax, diffusely stained (cytosolic) cytochrome *c*, and fragmented nuclei (per cent of total) were determined.

Transient transfection into HEK 293T cells and analysis by X-Gal staining. 3×10^5 human embryonic kidney (HEK) 293T cells grown on 35-mm plastic plates were transfected with either 1 μ g each of *bax*/pcDNA3, β -gal/pcDNA3 and pcDNA3 or *bax*/pcDNA3, β -gal/pcDNA3 and *bcl-2*/pcDNA3 using 15 μ g Superfect. After various times, cells were stained with 5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside (X-Gal) and examined by phase-contrast microscopy as described⁷. To determine the transfection efficiency, we counted trypanized blue and white cells in a Neubauer chamber. To determine the extent of apoptosis, we counted blue cells that were round and blebbing on 20 randomly selected fields under a Nikon inverted microscope at a magnification of $\times 400$ (phase contrast).

Protein extraction and western blotting. At 6–36 h post-transfection, HEK 293T cells were either directly lysed in a Tris/SDS buffer as described¹² (total extract) or disrupted by Dounce homogenization using buffer STE (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 2 μ g ml⁻¹ leupeptin, 1 mM PMSF, 1 μ g ml⁻¹ pepstatin), followed by ultracentrifugation to separate cytosol and membranes. Equal amounts of protein were subjected to immunoblotting using either mouse monoclonal anti-cytochrome *c* 7H8.2C12 (Pharmingen) or

rabbit polyclonal anti-caspase-3 (gift from D. Nicholson¹³) antibodies, followed by horseradish peroxidase-coupled secondary antibodies (Jackson Laboratories) as described¹². Immunodetection was by enhanced chemiluminescence (Pierce). Cytochrome *c* was quantified by densitometric scanning of autoradiographs.

DNA fragmentation. At 6–36 h post-transfection, HEK 293T cells of a 35-mm well were washed three times in phosphate-buffered saline and then immediately lysed in phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase containing nucleic acids was treated with RNase and loaded onto a 2% agarose gel containing ethidium bromide in order to visualize DNA fragmentation.

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The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*

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*Trypanosoma brucei*¹ is a unicellular parasite transmitted between African mammals by tsetse flies. *T. brucei* multiplies freely in the bloodstream of many different mammals, and survives by antigenic variation of the main component of its surface coat, variant surface glycoprotein (VSG)^{2,3}. Trypanosomes take up transferrin through a heterodimeric transferrin receptor^{4–9}, the genes for which are expressed in telomeric expression sites along with the VSG gene. There are up to 20 of these expression sites per trypanosome nucleus^{3,10–15}, but usually only one is active at a time. Different expression sites encode transferrin receptors that are similar but not identical¹⁶. Here we show that these small differences between transferrin receptors can have profound effects on the binding affinity for transferrins from different

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mammals, and on the ability of trypanosomes to grow in the sera of these mammals. Our results suggest that the ability to switch between different transferrin-receptor genes allows *T. brucei* to cope with the large sequence diversity in the transferrins of its hosts¹⁷.

The transferrin receptor (Tf-R) of mammals consists of two identical subunits of relative molecular mass 90,000 (M_r 90K) with a single transmembrane segment¹⁸, whereas that of *T. brucei* is a heterodimer^{4-7,19} (Fig. 1) anchored in the membrane by the carboxy-terminal glycosylphosphatidylinositol (GPI) tail of the subunit encoded by expression site associated gene (ESAG) 6. The ESAG 7 subunit has substantial homology with the ESAG 6 subunit, but lacks a GPI tail or any other obvious means of membrane attachment. It presumably remains attached to the trypanosome surface by holding on to the ESAG 6 subunit⁷.

The Tf-Rs encoded by different expression sites differ by a hypervariable region that looks as if it has been selected for

variability¹⁶. This raised the possibility that these regions serve as immunodominant epitopes in the Tf-R. By changing the expression site transcribed, the trypanosome would be able to avoid interference by anti-receptor antibodies²⁰. To test this hypothesis, we immunized mice with various forms of Tf-R. Although anti-receptor antibodies were formed, they had no effect on the multiplication of trypanosomes making this receptor.

This result was unexpected because it has previously been reported⁷ that the Tf-R of the EATRO 1125 strain of *T. brucei* had a low affinity for bovine transferrin and that there was substantial inhibition of transferrin uptake by anti-receptor antibodies⁷. We therefore determined the binding affinity of bovine transferrin to the Tf-R of our 221 expression site⁴. Rather than use bloodstream trypanosomes, in which the receptor is in the flagellar pocket and is poorly accessible at low temperature, we used the receptor reconstituted in insect-form trypanosomes, which spreads over the entire trypanosome and is not internalized⁴. Scatchard analysis of bovine

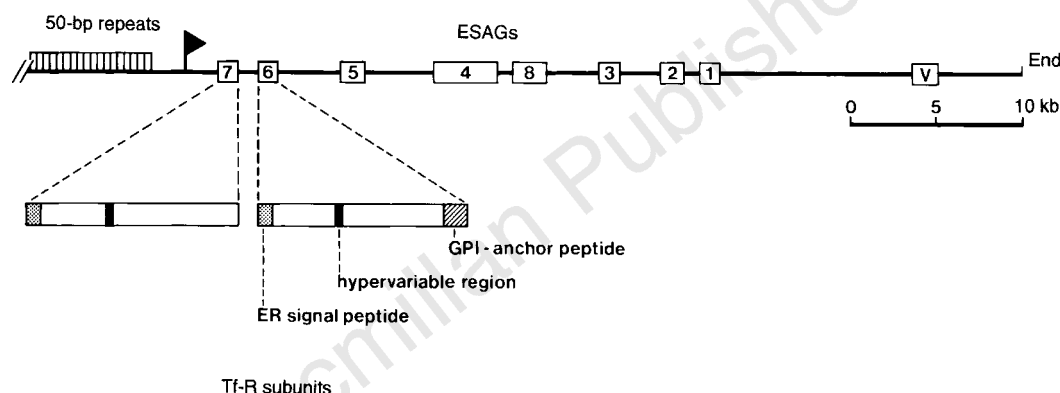


Figure 1 Schematic representation of a trypanosomal variant surface glycoprotein (VSG) gene expression site. Modelled after Revelard *et al.*³⁰ and Lips *et al.*¹². The flag indicates the promoter, the numbers in the boxes indicate the expression

site-associated genes (ESAGs)¹⁰⁻¹², V indicates the VSG gene, and End indicates the chromosome terminus. The enlargements show features of ESAG 6 and ESAG 7, which encode subunits of the Tf-R.

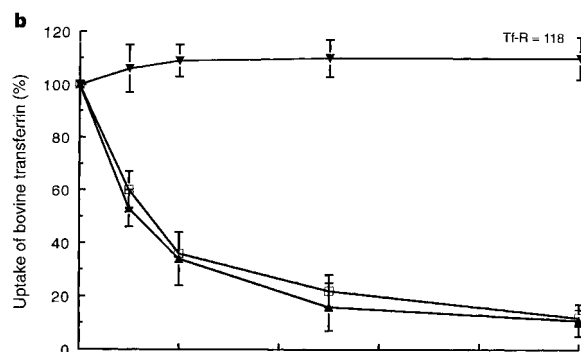
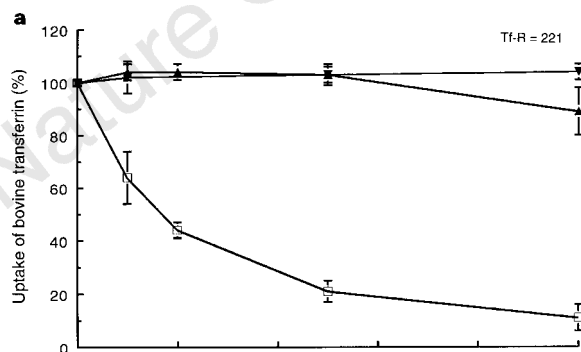
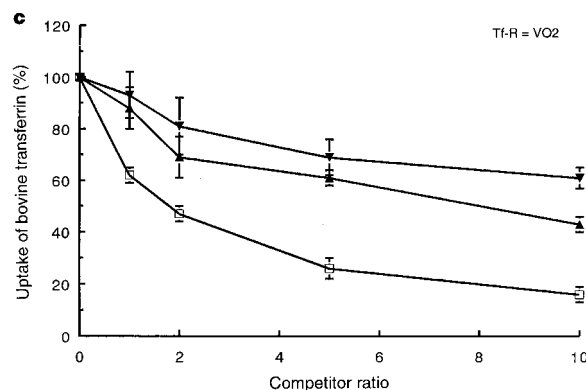


Figure 2 Effect of competing transferrins from various mammals on the uptake of ⁵⁵Fe-labelled bovine transferrin by three different *T. brucei* 427 variants. Each variant expresses a different expression site and therefore also a different Tf-R. **a**, Variant 221a (MITat 1.2); **b**, variant 118a (MITat 1.5); **c**, variant VO2 (the variant selected on canine serum). Data are from three experiments, and show mean $\pm$ s.d. Transferrin uptake was competed with holo-bovine transferrin ($\square$) holo-human transferrin ($\blacktriangle$) and holo-canine transferrin ($\blacktriangledown$). Uptake of transferrin was started by the addition of a mixture of [⁵⁵Fe]-transferrin (10 μ g ml⁻¹ final concentration) and various amounts of iron-saturated cold transferrins.



transferrin binding gave a single binding site for transferrin and a K_d of 2.6 nM (Table 1), that is, 300-fold lower than the EATRO 1125 receptor⁷. A low K_d (4 nM) had also been reported¹⁹ for a Tf-R encoded by the active expression site in variant 118a (MITat 1.5).

The high affinity of the 221 Tf-R for transferrin explained the poor competition of anti-receptor antibodies with transferrin for the Tf-R, but raised a new problem: how can a single Tf-R tightly bind transferrins from a diverse range of mammals? Transferrins have diverged considerably in mammalian evolution; for example, human and bovine transferrins differ by 30% in amino-acid sequence¹⁷, and bovine transferrin binds poorly to the human Tf-R^{21,22}. We therefore tested whether transferrins of other mammals could compete with bovine transferrin for uptake by bloodstream trypanosomes. Murine transferrin competed as effectively as bovine transferrin itself (data not shown), but human and canine transferrins were poor competitors (Fig. 2a). How then do trypanosomes survive in human or dog serum?

The answer to this question came from an analysis of variants of *T. brucei* 427 that use VSG gene expression sites other than the 221 site. Results with variant 118a, in which the 118 site (also known as the dominant expression site) is active, are shown in Fig. 2b. With this receptor, human transferrin competed as effectively as bovine transferrin itself, but canine transferrin was still a poor competitor. To find a Tf-R that binds canine transferrin, we transferred trypanosome variant 221a (MITat 1.2), which makes the 221 Tf-R, to medium supplemented with canine serum. On bovine serum, variant 221a grows well with only rare switches of expression site. On canine serum, however, most of the trypanosomes stopped dividing (Fig. 3). The trypanosomes that grew out produced a Tf-R that bound canine transferrin more efficiently (Fig. 2c). These trypanosomes had switched, in several independent experiments, to the VO2 expression site²³. Addition of small amounts of bovine holo-transferrin ($0.04 \mu\text{g ml}^{-1}$) to the canine serum-supplemented medium resulted in normal growth of the 221a variant, and no switched trypanosomes could be isolated from this culture. Growth of 221a trypanosomes could also be achieved by adding human or canine holo-transferrin, although higher amounts of these proteins were required, 6 and $100 \mu\text{g ml}^{-1}$, respectively. It seems that the absence of a suitable transferrin source drives the selection for switched trypanosomes in this system.

The analysis of the 118, 221 and VO2 Tf-Rs reconstituted in insect-form trypanosomes (Table 1) shows that they all bind bovine transferrin efficiently, whereas binding of human and canine trans-

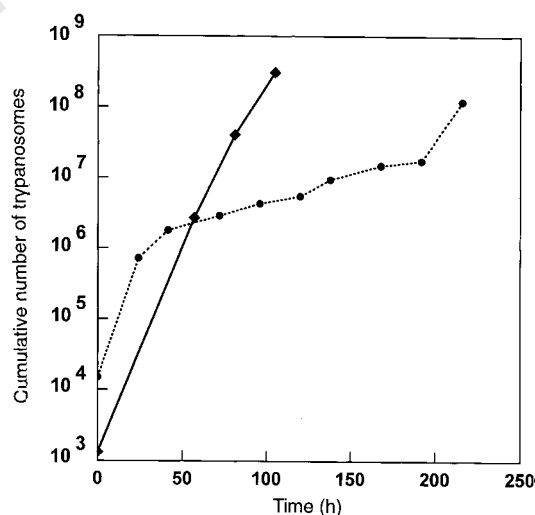


Figure 3 Growth characteristics of bloodstream trypanosome variant 221a on HMI-9 medium supplemented with 10% fetal calf serum or 10% canine serum. Calf serum, ♦; canine serum, ●.

Table 1 Binding of bovine, human and canine transferrin to various Tf-Rs reconstituted in insect-form trypanosomes

Transferrin receptor	Dissociation constants (nM)		
	Bovine transferrin	Human transferrin	Canine transferrin*
221	2.6 ± 0.7	31 ± 20	
118	2.6 ± 0.5	5.3 ± 1.4	
VO2	13 ± 3	90 ± 16†	159 ± 35†

* We could not detect any binding of canine transferrin to the Tf-R expressed in 221 and 118 trypanosomes.

† The low-affinity binding of human and canine transferrin to the VO2 receptor did not allow direct K_d determinations. Therefore these K_d values were calculated from the K_d of bovine transferrin and the competition of bovine transferrin uptake by human and canine transferrin.

ferrin occurs with very different K_d values. Canine transferrin binds to the VO2 receptor, but with a relatively low affinity (Table 1), whereas the affinity of the 221 and 118 Tf-Rs for canine transferrin was too low to be determined accurately. These data confirm the results of transferrin uptake by bloodstream trypanosomes. The reconstitution system also made it possible to make chimaeric receptors, and show that high-affinity binding of human transferrin by the 118 Tf-R is associated with the ESAG 7 subunit (data not shown).

These results provide a new explanation for Tf-R variability. *T. brucei* has colonized a large range of mammals and has therefore been forced to devise a way of taking up different transferrins efficiently without being troubled by the anti-Tf-R antibodies known to arise in a chronic infection²⁴. The trypanosome seems to have solved this problem by creating a separate high-affinity receptor for different groups of mammals, giving anti-receptor antibodies no chance. Indeed, antibodies directed against the 221 Tf-R inhibited the uptake of bovine transferrin less than that of human transferrin ($32 \pm 5\%$ compared with $70 \pm 6\%$, $n = 4$). The example of the Tf-R suggests that the other ESAGs could also code for variant trypanosome components that are optimized for certain mammalian hosts. By switching rapidly between expression sites early in infection²⁵, the trypanosome population could generate a variant most suitable for that host²⁶. □

Methods

Trypanosomes. The trypanosomes used are from strain 427 of *Trypanosoma brucei brucei*². Bloodstream-form trypanosomes 221a (MITat 1.2) and 118a (MITat 1.5) were grown in HMI-9 medium²⁷ supplemented with 10% fetal calf serum or with 10% canine serum. Insect-form trypanosomes were grown in semidefined medium²⁸.

Transferrin uptake and competition. Transferrin uptake experiments were performed according to ref. 4. Transferrin uptake was started by adding a mixture of ⁵⁵Fe-saturated bovine transferrin ($10 \mu\text{g ml}^{-1}$ final concentration) and various amounts of iron-saturated cold transferrins and determined after 40 min at 37 °C. The uptake of ⁵⁵Fe-labelled bovine transferrin without competitor was similar in all three variants and equivalent to 6.2 ng transferrin per 10^6 cells per h (± 1.2 ng).

Antibody competition with transferrin uptake. Antibodies directed against the 221 Tf-R were obtained from a rabbit immunized with intact 221 Tf-R isolated by affinity chromatography⁹ from insect-form trypanosomes expressing this receptor. Immunoglobulins were purified with a protein A-agarose affinity column and used in the competition assay in a sixfold excess to transferrin, which is the transferrin: immunoglobulin ratio present in plasma. Uptake of transferrin was determined as described above at different time points.

Expression of Tf-R in insect-form trypanosomes. ESAG 6 and ESAG 7 of the 118a and VO2 expression sites were amplified using reverse transcription-polymerase chain reaction (RT-PCR) and cloned in small polycistronic transcription units⁴. The constructs were targeted into a ribosomal array and placed under control of the ribosomal DNA promoter⁴.

Transferrin dissociation constant. Bovine and human transferrins (Sigma) were FITC-labelled as described²⁹. Equilibrium measurements for FITC-

transferrin binding to insect-form trypanosomes expressing ESAG 6 and ESAG 7 of different expression sites were performed using FACSscan flow cytometry (Becton Dickinson). Insect-form trypanosomes were washed twice with semidefined medium²⁸ in which serum was replaced by 0.5% bovine serum albumin and incubated in this medium for 2 h at room temperature with different concentrations of FITC-labelled transferrin. Fluorescence of cells expressing TF-R was corrected for fluorescence of wild-type trypanosomes incubated with the same transferrin concentration. The K_d was determined by Scatchard analysis.

Analysis of switched trypanosomes. The variant selected on canine serum was established to be VO2 (ref. 23) by sequence analysis of the cDNA of the newly expressed variant surface glycoprotein and pulsed field gel electrophoresis.

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Structure of the V δ domain of a human $\gamma\delta$ T-cell antigen receptor

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Antigen recognition by T lymphocytes is mediated by cell-surface glycoproteins known as T-cell antigen receptors (TCRs). These are composed of α and β , or γ and δ , polypeptide chains with variable (V) and constant (C) regions. In contrast to $\alpha\beta$ TCRs, which recognize antigen only as peptide fragments bound to molecules of the major histocompatibility complex (MHC), $\gamma\delta$ TCRs appear to recognize proteins directly, without antigen processing, and to recognize MHC molecules independently of the bound peptide^{1–4}. Moreover, small phosphate-containing non-peptide compounds have also been identified as ligands for certain $\gamma\delta$ T cells^{5,6}. These studies indicate that antigen recognition by $\gamma\delta$ TCRs may be fundamentally different from that by $\alpha\beta$ TCRs. The three-dimensional structures of several $\alpha\beta$ TCRs and TCR fragments^{7–10}, and their complexes with peptide–MHC or superantigens^{9–11}, have been determined. Here we report the crystal structure of the V δ domain of a human $\gamma\delta$ TCR at 1.9 Å resolution. A comparison with antibody and $\alpha\beta$ TCR V domains reveals that the framework structure of V δ more closely resembles that of V H than of V α , V β or V L (where H and L refer to heavy and light chains), whereas the relative positions and conformations of its complementarity-determining regions (CDRs) share features of both V α and V H . These results provide the first direct evidence that $\gamma\delta$ TCRs are structurally distinct from $\alpha\beta$ TCRs and, together with the observation that the CDR3 length distribution of TCR δ chains is similar to that of immunoglobulin heavy chains¹², are consistent with functional studies suggesting that recognition of certain antigens by $\gamma\delta$ TCRs may resemble antigen recognition by antibodies^{1–3}.

We present the crystal structure of the V δ domain of a human $\gamma\delta$ TCR designated ES204 (V γ 1.3–J γ 1.3, V δ 3–D δ 2–J δ 1) specific for the MHC class I molecule HLA-A2 (ref. 13). The structure was determined by multiple isomorphous replacement using engineered heavy-atom binding sites and refined to a crystallographic R-value of 17.4% for all data between 6.0 Å and 1.9 Å resolution. Crystallographic statistics are reported in Table 1; a representative portion of the $2F_o - F_c$ electron density map is shown in Fig. 1a.

The TCR V δ domain is structurally homologous to the V domains of both immunoglobulins and $\alpha\beta$ TCRs with CDR loops disposed to form part of the antigen-combining site (Fig. 1b). A sequence alignment, derived from superposing the crystal structure of ES204 V δ onto antibody and $\alpha\beta$ TCR V region structures, is given in Fig. 2. When V δ is superposed onto nine representative V H domains by overlapping 89 structurally equivalent framework residues, an average root-mean-square difference (r.m.s.) in α -carbon positions of 1.1 Å is obtained; a similar comparison with nine V L domains (85 framework residues overlapped) gives a r.m.s. difference of 1.7 Å. The r.m.s. difference is 1.4 Å with respect to three V α domains^{8–10} for 85 framework residues and 1.6 Å with respect to

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three V β domains^{7,9,10} for 83 residues. The nearest match is to the V H domain of a human IgM immunoglobulin¹⁴ (r.m.s. difference of 0.9 Å). The framework structure of the ES204 V δ domain therefore appears to be closest to that of V H , even though V δ regions are most similar to V α in terms of amino-acid sequence^{15,16} and are encoded in the same genetic locus as V α ¹⁷. In addition, certain V δ s have been found to rearrange to either J δ or J α gene segments¹⁶.

Superpositions of the ES204 V δ structure onto representative antibody and TCR V domains are shown in Figure 3. As can be seen (Fig. 3a), the largest departure from the V α framework occurs in the position of the c' β -strand (see ref. 18 for strand nomenclature). In all three known V α structures⁸⁻¹⁰, the c' strand is associated with the d strand of the adjacent β -sheet through a number of backbone-backbone hydrogen bonds, resulting in a β -strand topology different from that of antibody V domains. In V δ , the c' strand is hydrogen-bonded to the c' strand in the same β -sheet, as in V L and V H domains (Fig. 3c, d) and in V β (Fig. 3b). This structural difference between V δ and V α is due, at least in part, to an insertion of three residues at positions 57A–59 (numbered as in ref. 15) compared with 2C V α and of four residues compared with 1934.4 V α and A6 V α (Fig. 2). The insertion, which makes the sequence of human V δ 3 in this region equal in length to that of V L or V H rather than V α , results in a displacement of the c' β -strand away from the d strand and toward the c' strand. Other V δ domains may or may not display the same folding topology as ES204 V δ . For example, mouse V δ 5 very probably folds like human V δ 3, because these two V δ

segments share 72% sequence identity and have the same number of inserted residues at positions 57A–59. However, human V δ 1 and V δ 2 are each three residues shorter in this region¹⁵ and may display the same c' strand switch as V α . It is also possible that certain V δ regions lacking an insertion at positions 57A–59 on the basis of current sequence alignments^{15,16} may in fact fold analogously to V L domains, which have the same β -strand topology as V H , despite being up to five residues shorter in the region 50–60. In the case of V L , the length of the c' strand is maintained at the expense of the CDR2 loop (Fig. 2) with the result that this CDR does not extend as far toward the other CDRs as in V H domains (Fig. 3d). Deciding between these possibilities will require determining the three-dimensional structure of additional V δ domains.

A consequence of the c' strand switch in V δ relative to V α is that the main chain of V δ CDR2 is almost perpendicular to that of V α CDR2 and nearly coincident with that of V H CDR2 (Fig. 4a, b). In contrast, the relative positions of CDR1 and CDR3 are nearly identical to those in V α ⁸⁻¹⁰. Thus, the overall topology of that portion of the ES204 combining site formed by the V δ CDRs displays key features of both antibody V H and TCR V α domains. The V δ CDR2 superposes onto the V H CDR2s of antibodies NEW, HyHEL-10 and Pot with r.m.s. differences in α -carbon positions of 1.0, 1.0 and 0.7 Å, respectively, and onto the V α CDR2s of TCRs 1934.4, 2C and A6 with r.m.s. differences of 1.6, 1.7 and 2.4 Å, respectively. The tight ES204 CDR2 loop has a type VI β -turn conformation, with Asp 52 and Ser 53 protruding from the surface

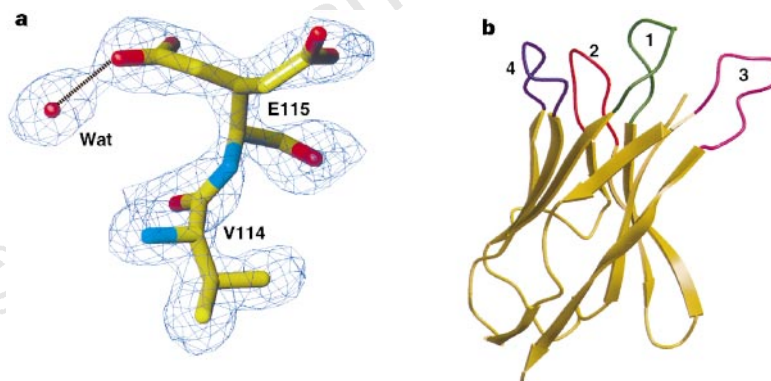


Figure 1 Structure of the TCR V δ domain (V δ 3-D δ 2-J δ 1). **a**, Electron density from the final 1.9 Å 2F_o - F_c map, contoured at 1 σ in the region of the C terminus of V δ showing two conformations for the side chain of Glu 115 and a hydrogen-bonded

water molecule (Wat). Carbon, nitrogen, and oxygen atoms are coloured yellow, blue and red, respectively. **b**, Ribbon diagram of ES204 V α . The CDR loops are numbered 1, 2 and 3; HV4 is labelled 4.

	1	10	20	30	40	50	
V δ ES204	DKTSSPDQTVASGSEVVL	LT	YDTV-YSNPDL	FYRIRPDYSFQVF	YGD-D-SRSE		
V α Pot	EVHLLS-SGGNLVPGGSLR	SC	AASGFTNFVMSVR	APGKGLWVSGVFG	SGGGNTD		
V α 1934.4	DSSTTEGQVASEEDFLTH	NY	SAS--GYPALFVYVYP	GGPQFLFRAS-RDKEKG			
V α 2C	QSVTPDARVTVSEGLQLR	KY	SYSS--ATPYLFVYVYP	RQGLQLLLKYY-SGDPVV			
V α A6	KEPEINSGLSVPEGAIASN	TY	SDR--GSQSFVYRQY	SGKSPELIMSIY-SN-GDK			
V β 14.3.d	ANTSPRNKVAVTGGKVT	SN	QNTNN--HNNMYRQDT	GHGLRLIHYSY-GAGSTE			
V β 2C	EAAVTPSRNKVAVTGGKVT	SN	QNTNN--HNNMYRQDT	GHGLRLIHYSY-GAGSTE			
V β A6	QNTTPKFQVLKTGQSMTC	Q	AQDMN--HEYMSYRQD	PGMGLRLIHYSV-GAGITD			
V L Pot	DIQMTSPSSLSASVGDRT	TS	QASQD-ISN-YLALYQ	KPGKAPELRIYDA--SN			
							c'
	57ABCDE	60	70	80	90	101	110
V δ ES204	GADFTQGRFSVKHILTKAFH	V	SPVRTEDSATYERF	TL--PPPTDKLIF	GRKGRVTV	EP	
V α Pot	YADAVKGRFTITRDNKNTLY	Q	MNSLRADTAIYAK	HRVSYVLTGFD	SGQQLVTVSS		
V α 1934.4	SS--R--GFEATYNKEATSFH	Q	ASVQESDAVYALSE	--NYGNEKIT	YAGAKLT	KP	
V α 2C	QG--VN--GFEAEFSKNSSPH	R	ASVHWSDAVFAVSG	--FASA-LTPG	SKVILP		
V α A6	ED--G--RFTAQLNKASQYVSL	L	RDSQPSDSATLCAV	TT--D-SWGKLG	AGACQVVT	TP	
V β 14.3.d	KGD-IPDGYKASRPSQ-EQF	S	ILELATPSQTSVTF	ASGGG-RGSYAEQF	GGPGRRLT	LE	
V β 2C	KGD-IPDGYKASRPSQ-ENF	S	ILELATPSQTSVTF	ASGGG----	GTLYAGAC	RLT	LE
V β A6	QGE-VPNGYVNSRST-EDF	P	RLLSAAPSQTSVTF	ASRPLAGGRPEQY	GGPGRRLT	TE	
V L Pot	LETGVPSRFSGSGSG--TD	F	FTFTSSLPQED	IATYHQQYQN----	LPLTSG	SKVD	KR
							c'

Figure 2 Alignment of the ES204 V δ sequence with immunoglobulin and TCR V domain sequences based on structural information. The α -carbon skeletons were first optimally superposed. Sequences were then manually adjusted to minimize the number of gaps while respecting the structural similarity. Numbering of the V δ sequence is according to ref. 14. Colours are as follows: CDR1 (green),

CDR2 (red), CDR3 (pink) and HV4 (blue). CDR boundaries are defined as in refs 7–9 and 19. Highly conserved or invariant residues are boxed in red; less conserved residues are highlighted in blue or blue-green. The position of the c' strand is indicated.

of the binding site (Fig. 4c). The V δ CDR2 appears to be stabilized by four intraloop backbone–backbone hydrogen bonds (Glu 56 N–O Tyr 49, Glu 56 O–N Tyr 49, Arg 54 O–N Asp 51, and Ser 53 N–O Asp 51), by three additional intraloop hydrogen bonds with the side chain of Asp 51 (Asp 51 O δ 1–N Ser 53, Asp 51 O δ 1–O γ Ser 53, and Asp 51 O δ 1–N Arg 54), and by two backbone–backbone hydrogen bonds with CDR1 residue Leu 33 (Leu 33 N–O Gly 50 and Leu 33 O–N Gly 50).

Although the framework structure and CDR2 loop conformation of V δ 3 are most like that of V H , its CDR1 conformation clearly resembles that of V α . The ES204 V δ CDR1 superposes onto the CDR1 of V α domains 1934.4, 2C and A6 with r.m.s. differences in α -carbon positions of 1.0, 1.0 and 1.9 Å, respectively. A similar comparison with the CDR1s of nine V L s, nine V H s and three V β s^{7,9,10} gives r.m.s. differences of 2.2, 2.1 and 1.8 Å, respectively. In contrast to V L and V H domains, whose CDR1s are mainly stabilized by the hydrophobic side chain of residue 29 which intercalates between two β -pleated sheets, V δ 3 CDR1 is mainly stabilized by hydrophobic interactions between highly conserved residues Tyr 24 and Leu 33, as in the case of V α ^{8–10} (Fig. 4d). The V δ CDR1 is further stabilized by hydrogen bonds to CDR2 residues Tyr 49 and Asp 52 (Asp 32 O δ 1–O γ Tyr 49, Asn 30 O δ 1–N Asp 52) and to framework residues Asp 1, Lys 2 and His 64 (Asp 1 O δ 2–O γ 1 Thr 26, Lys 2 N–O Asp 25, Lys 2 O–N Asp 25, Tyr 24 O η –N ϵ 2 His 64). The conservation of hydrophobic residues at positions 24 and 33 in both V α

and V δ sequences suggests that the CDR1 conformation described here is common to most α and δ chains and represents a canonical structure¹⁹ for this CDR loop.

An analysis of the CDR3 length distribution of antibodies and TCRs has revealed that the length profiles of $\gamma\delta$ TCRs are more similar to those of antibodies than of $\alpha\beta$ TCRs¹². In particular, TCR δ and immunoglobulin H chains have the longest and most variable CDR3 lengths (6–21 residues for δ chains and 1–25 residues for H chains), whereas the CDR3s of α , β , γ and light chains are notably shorter and less variable. However, in terms of its relative position in the TCR combining site, V δ CDR3 resembles V α CDR3 more than V H CDR3 (Fig. 4a, b): unlike V H CDR3s, which are directed away from the core of the domain (Fig. 3c), V δ CDR3 folds back toward CDR1 and CDR2, as in V α (Fig. 3a). The ES204 CDR3 loop is 10 residues long, which is about the median length for δ chains¹², and forms part of a relatively flat binding surface (Fig. 1b), as observed in $\alpha\beta$ TCRs^{9,10}. Longer V δ CDR3s are likely to project from the surface of the domain, thereby creating protuberances or crevices which may facilitate the binding of $\gamma\delta$ TCRs to a wider variety of antigenic surfaces than $\alpha\beta$ TCRs. The ES204 CDR3 consists of a type II β -turn with an unusual repeat of three consecutive proline residues (Pro 98–Pro 99–Pro 100–Thr 101) followed by a classic γ -type turn (Thr 101–Asp 102–Lys 103) at its tip (Fig. 4e). This CDR is stabilized by a number of intraloop backbone–backbone and backbone–side-chain hydrogen bonds: Leu 97 N–O Lys 103, Leu 97 O–N

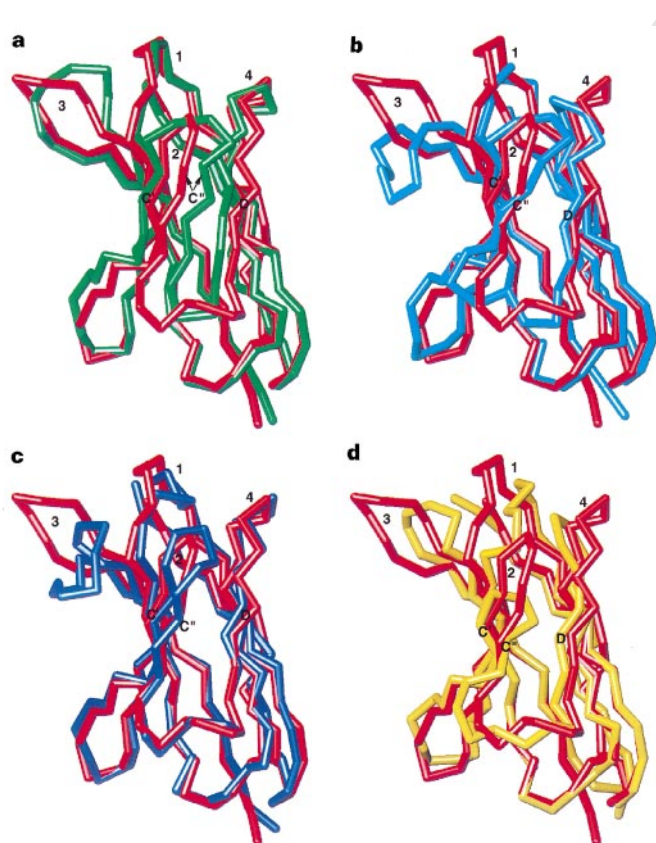


Figure 3 Comparison of V δ with V α , V β , V H and V L structures. **a**, α -Carbon diagram of ES204 V δ (red) superposed onto 1934.4 V α (green). **b**, V δ superposed onto 14.3d V β (blue). **c**, V δ superposed onto Pot V H (purple). **d**, V δ superposed onto Pot V L (yellow).

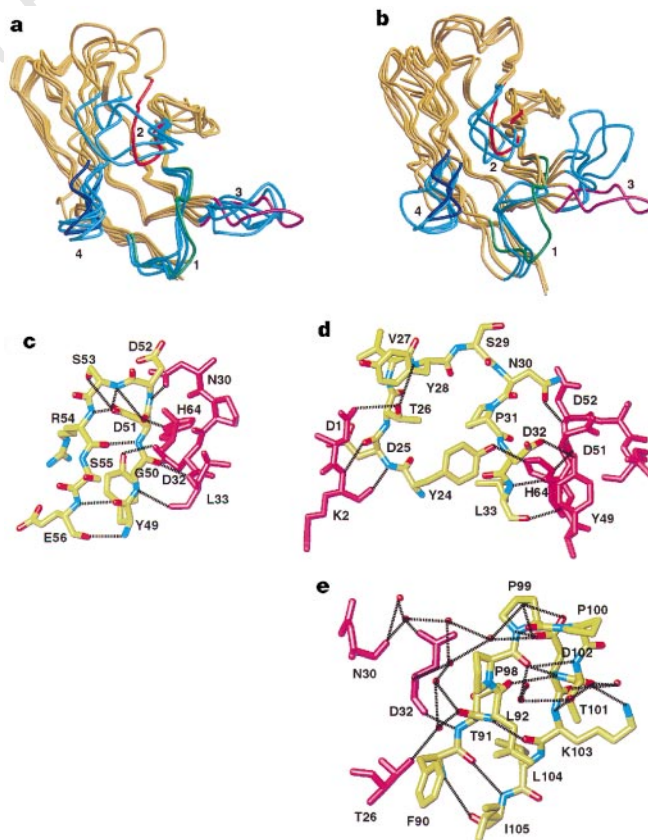


Figure 4 The antigen-binding loops of ES204 V δ . **a**, Overlap of ES204 V δ with three V α s (refs 7–9) to show relative dispositions of CDRs. The V δ and V α CDRs are labelled 1, 2 and 3; HV4 is numbered 4. V δ CDR1 is coloured green, CDR2 red, CDR3 pink and HV4 purple; the corresponding regions of V α are blue. **b**, Overlap of ES204 V δ with three V H s (New, HyHEL10 and Pot; PDB accession numbers 3fab, 3Hfm and 1igm, respectively). The CDR and HV4 loops are labelled and coloured as in **a**. **c**, V δ CDR2, residues 49 to 56. Hydrogen bonds are dotted black lines. Atoms are coloured as in Fig. 1. Residues that interact with CDR are shown in pink. **d**, CDR1, residues 24 to 33. **e**, CDR3, residues 91 to 105. Bound water molecules are shown as red spheres.

Table 1 Data collection, phasing and refinement statistics

a Data collection and phasing statistics	Native 1	Native 2	Pt1* (Q10M)	Pt2* (L33M)	Pt3* (R54M)
Resolution limit (Å)	2.23	1.90	2.65	2.56	2.17
Observations	27,416	44,342	24,518	31,457	82,049
Unique reflections	9,214	13,905	5,384	6,652	11,307
Completeness (%)†	84.9/36.2	78.1/53.7	81.1/52.5	85.7/47.7	95.2/70.9
$R_{\text{sym}}^{\ddagger}$ (%)	7.7	8.1	6.0	9.3	7.1
$R_{\text{iso}}^{\ddagger}$ (%)			13.0	18.2	33.1
Working resolution (Å)			15–3.0	15–3.0	15–3.0
Heavy-atom sites			2	2	3
(PP)§ (acentric/centric)			1.56/1.20	0.92/0.71	0.89/0.71
$R_{\text{cullis}}^{\S}$ (acentric/centric)			0.72/0.61	0.86/0.82	0.88/0.82
$R_{\text{anomalous}}^{\S}$			0.93	0.95	0.87
Mean FOM§				0.62	
b Refinement statistics					
Resolution range (Å)		6–1.9	r.m.s. deviations from ideality		
$R_{\text{merge}}^{\parallel}$ (%) Native 1 and 2		9.3	bond lengths (Å)		
Reflections		14,814	bond angles (°)		
Completeness (6–1.9 Å/2.0–1.9 Å, %)		83.3/53.7	impropers (°)		
$R_{\text{factor}}^{\parallel}$ (%)		16.1	dihedrals (°)		
$R_{\text{free}}^{\parallel}$ (%)		22.4	Average atomic B factors (Å ²)		
$R_{\text{all}}^{\parallel}$ (%)		17.4	main chain		
Atoms in model			side chain		
protein (non-hydrogen)		1,894	water		
water		309			

* Crystals with methionine substitutions at positions 10, 33 and 54 were soaked in 5 mM, 10 mM and 10 mM K₂PtCl₆ for 12 h, 18 h and 2 days, respectively. †Number after slash is for the highest resolution shell. ‡ $R_{\text{sym}} = \sum |I_{\text{hkl}} - \langle I_{\text{hkl}} \rangle| / \sum \langle I_{\text{hkl}} \rangle$, where $\langle I_{\text{hkl}} \rangle$ is the mean intensity of all reflections equivalent to reflection hkl by symmetry; $R_{\text{iso}} = \sum |F_{\text{PH}} - F_{\text{P}}| / \sum F_{\text{P}}$, where F_{PH} is the derivative amplitude and F_{P} is the native amplitude. §(PP) (phasing power) = $\sum |F_{\text{H(calc)}}| / (\sum |F_{\text{H(obs)}}| - F_{\text{H(calc)}})$; $R_{\text{cullis}} = \sum |F_{\text{H(obs)}} - F_{\text{H(calc)}}| / \sum |F_{\text{H(obs)}}|$ where F_{H} is the heavy-atom structure factor; $R_{\text{anomalous}} = (\text{lack of closure}) / (\text{anomalous difference})$; FOM is figure of merit. ||Only 4.0 Å resolution data were used to calculate anomalous dispersion for this derivative. ¶ R_{merge} is defined as R_{sym} ; $R_{\text{factor}} = 100(\sum |F_{\text{o}}| - |F_{\text{c}}|) / \sum |F_{\text{o}}|$; 10% of reflections (1,481) were used for R_{free} ; (R_{all}) R_{factor} was calculated for the last cycle of refinement by using all data between 6 Å and 1.9 Å.

Asp 102, Pro 98 O–N Asp 102, Pro 98 O–N Thr 101, Pro 99 N–Oδ2 Asp 102, Thr 101 O–N Lys 103, and Thr 101 O–Nζ Lys 103. Additional stabilization derives from an extensive network of water-mediated hydrogen bonds which include Thr 26 Oγ1–H₂O–O Thr 96–H₂O–H₂O–N Asp 32, Arg 54 O–H₂O–Nδ1 His 64–H₂O–H₂O–Oγ Ser 56, and Asp 51 Oδ2–H₂O–Nγ2 Asn 30. These bound water molecules not only help maintain the conformations of the CDR loops of this Vδ, but also link them tightly together to form part of the antigen-combining site of the ES204 TCR. Bound water molecules have also been observed in the combining site of antibodies, where they act to increase complementarity in the interface with antigen²⁰.

The Vδ loop comprising residues 64–71, which is analogous to the fourth hypervariable region (HV4) of V_H and Vβ, is directed away from CDR1 and CDR2 as in V_H and Vα^{8–10} domains. In V_L and Vβ domains, by contrast, this loop folds toward CDR1 and CDR2. Because Vδ HV4 forms part of a relatively flat continuous surface with the CDRs, it could potentially be involved in interactions with antigen, as is Vα HV4 in the A6 TCR–Tax/HLA–A2 complex¹⁰ and the HV4-equivalent region of V_L in an idiotope-anti-idiotope antigen–antibody complex²¹.

The ES204 Vδ domain exists as a homodimer both in solution²² and in the crystal structure. The hydrophobic surface of Vδ that, by analogy to antibodies and αβ TCRs^{9,10}, should form the interface with Vγ in the γδ heterodimer does indeed contact the same surface of a neighbouring Vδ chain in the crystal lattice. However, instead of forming an antibody Fv-like structure, the two V domains pack head-to-tail such that their CDR loops appear on opposite sides of the dimeric aggregate, as in the case of the 14.3d TCR β-chain structure⁷. Most of the residues at this interface are equivalent by homology to those involved in VαVβ and V_LV_H dimerization, which indicates that the geometry of VγVδ association is likely to be similar to that of VαVβ and V_LV_H. However, the putative Vγ-contacting surface of ES204 Vδ is considerably more hydrophobic than the corresponding surface of Vα domains^{8–10} and includes the following substitutions relative to 1934.4 Vα: Gln 37 → Ile 38, Glu 41 → Tyr 42, Pro 43 → Phe 44, Arg 48 → Tyr 49 and Glu 93 → Leu 92 (Fig. 2). The change of Gln 37 in Vα to Ile 38 in Vδ3 would result in the loss of a pair of side-chain–side-chain hydrogen bonds with Vγ Gln 39 in the γδ heterodimer that are invariant in VαVβ (and V_LV_H) structures^{9,10}. In other Vδ sequences, position 38 is

glutamine or lysine. There is a similar lack of conservation of Vγ Gln 39, which may also be arginine or lysine, but which is nearly always glutamine in Vβ. This variability at positions Vγ 39 and Vδ 38 may result in the preferential pairing of certain γ and δ chains, as observed in early fetal ontogeny²³.

It is becoming increasingly apparent that γδ T cells have different antigen recognition requirements from αβ T cells². Early studies showed that γδ T cells frequently lacked CD4 and CD8, implying that they might not be MHC-restricted¹⁷. This suggestion was supported by the finding that thymic development of γδ cells appeared normal in β₂-microglobulin-deficient and class II-deficient mice^{24,25}. The major reactivity that has been identified for human γδ T cells involves recognition of small aliphatic phosphate compounds from mycobacteria, such as isopentenyl pyrophosphate, that have no chemical relationship to peptides and do not require classic MHC class I or class II molecules for presentation⁶. In mice, γδ T cells have been identified that appear to recognize several protein antigens directly, without any requirement for processing. These antigens include the gI glycoprotein of herpes simplex virus⁴, MHC class II I–E^k (ref. 1), and the non-classical MHC class I TL10 molecule³. Moreover, recognition of I–E^k and TL10 was shown to be independent of bound peptide. These studies suggested that antigen recognition by γδ T cells may resemble antigen recognition by antibodies, which also recognize antigens directly and without processing, and that there may be significant structural differences between γδ and αβ TCRs. We have shown that the Vδ domain of an MHC class I-reactive γδ TCR incorporates key structural elements of both TCR and immunoglobulin V regions. Although its framework more closely resembles that of V_H than Vα, the conformations and relative positions of its CDRs share features of both Vα and V_H domains. Thus, Vδ CDR2 is nearly coincident with V_H CDR2, as is Vδ CDR1 with Vα CDR1. Furthermore, although the CDR3 length distribution of δ chains resembles that of H chains, Vδ CDR3 is oriented in the TCR combining site in a similar way to Vα CDR3. This particular combination of structural features from both TCR Vα and antibody V_H domains no doubt contributes to the unique antigen recognition properties of γδ TCRs. □

Methods

Protein expression and crystallization. The ES204 Vδ domain was expressed as a secreted protein in the periplasmic space of *Escherichia coli* and crystallized

in 0.65–0.75 M sodium citrate, 0.1 M sodium cacodylate, pH 6.5 (ref. 22). The crystals belong to the space group $P2_12_12_1$, with unit cell dimensions $a = 69.9 \text{ \AA}$, $b = 49.1 \text{ \AA}$, $c = 61.7 \text{ \AA}$, and contain one $V\delta$ homodimer per asymmetric unit. Heavy-atom binding sites were introduced by individually mutating residues Q10, L33 and R54 to methionine. All three mutant proteins crystallized under conditions similar to those for the wild type; mutant and wild-type $V\delta$ crystals are isomorphous.

Data collection and processing. X-ray diffraction data to $2.3 \text{ \AA}$ resolution were collected from one native crystal at room temperature on a Siemens multiwire area detector using a rotating anode generator and processed with the program XENGEN2.1 (ref. 26) (Table 1). Higher resolution ($1.9 \text{ \AA}$) data were collected at 4°C from one crystal at Brookhaven National Laboratory beamline X8C ($\lambda = 1.0 \text{ \AA}$) using a Siemens CCD detector and processed with MADNES (ref. 27). Native data from the two sources were merged using SCALEPACK (ref. 28) to yield a data set 83.3% complete at $1.9 \text{ \AA}$ ($R_{\text{merge}} = 9.3\%$). Crystals with methionine substitutions at positions 10, 33 and 54 were extensively washed with 1.6 M sodium phosphate, pH 7.0, before soaking in solutions of K_2PtCl_4 prepared in the same buffer. Derivative data were measured in-house on an area detector (Table 1).

Structure determination and refinement. The structure of $V\delta$ was solved by multiple isomorphous replacement combined with anomalous scattering (MIRAS) using the CCP4 suite of programs²⁹ and PHASES. One major platinum site in Q10M mutant crystals was identified by difference Patterson synthesis with PHASES (Table 1). The minor sites, as well as heavy-atom positions for mutants L33M and R54M, were located by difference Fourier syntheses using Q10M phases. Heavy-atom parameters were refined with VECREF and MLPHARE (ref. 29). Phases from MIRAS, including anomalous signals for the three derivatives to $3.0 \text{ \AA}$, were improved by solvent flattening (40% solvent content) and histogram matching with DM (ref. 30). This gave interpretable electron density maps in which 78% of the polypeptide backbone of one $V\delta$ monomer could be unambiguously traced. The non-crystallographic 2-fold axis was identified by analysing the initial MIRAS model with the rigid superposition option of TURBO-FRODO. Non-crystallographic symmetry (NCS) molecular averaging with DM using a mask built from the initial model dramatically improved the electron density. All residues of one monomer could now be traced through iterative model building, SIGMAA-weighted phase combination, and X-PLOR refinement. The other monomer was then generated through pseudo-symmetry operations. Refinement of the structure with X-PLOR using the merged native data consisted of iterative rigid-body refinement, simulated annealing, positional refinement, and temperature factor (B) refinement, interspersed with model building using TURBO-FRODO. Tight NCS restraints were applied at the start of the refinement; these were gradually loosened as different side-chain conformations became apparent at high resolution. Water molecules were added if they corresponded to electron density peaks higher than 2σ in $F_o - F_c$ maps, made contact with at least one potential hydrogen bond donor or acceptor in the range 2.4 to $3.4 \text{ \AA}$, remained in electron density (1σ , $2F_o - F_c$) after subsequent refinement, and reduced R_{cryst} as well as R_{free} . Waters with B factors over $60 \text{ \AA}^2$ were removed from the model. The last cycle of refinement was performed against all data between 6 and $1.9 \text{ \AA}$. The final model contains all 232 protein residues (116 residues per monomer) and 309 water molecules with $R_{\text{all}} = 17.4\%$ and $R_{\text{free}} = 22.4\%$ for 10% of the reflections in the data set excluded from the refinement. Several side chains, including E115 of monomer 1 and K2, R39, R54, K108 and E115 of monomer 2, have two conformations. Two *cis*-prolines (P77 and P100) are found in the structure. No residues have disallowed ϕ or ψ angles and 88% have favoured angles.

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Correspondence and requests for materials should be addressed to R.A.M. (e-mail: mariuzza@indigo2.carb.nist.gov). Atomic coordinates have been deposited in the Brookhaven Protein Data Bank (accession number 1tvd).

Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein

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The yeast phosphatidylinositol-transfer protein (Sec14) catalyses exchange of phosphatidylinositol and phosphatidylcholine between membrane bilayers *in vitro*^{1,2}. *In vivo*, Sec14 activity is essential for vesicle budding from the Golgi complex³. Here we report a three-dimensional structure for Sec14 at $2.5 \text{ \AA}$ resolution. Sec14 consists of twelve α -helices, six β -strands, eight 3_{10} -helices and has two distinct domains. The carboxy-terminal domain forms a hydrophobic pocket which, in the crystal structure, is occupied by two molecules of *n*-octyl- β -D-glucopyranoside and represents the

phospholipid-binding domain. This pocket is reinforced by a string motif whose disruption in a *sec14* temperature-sensitive mutant results in destabilization of the phospholipid-binding domain. Finally, we have identified an unusual surface helix that may play a critical role in driving Sec14-mediated phospholipid exchange. From this structure, we derive the first molecular clues into how a phosphatidylinositol-transfer protein functions.

Sec14 (304 amino-acid residues M_r 35K) has primary sequence homology to a growing number of proteins such as mammalian retinaldehyde- and α -tocopherol-binding proteins^{4,5}, a noncatalytic domain of the human MEG2 protein tyrosine phosphatase⁶ and others⁷. The data suggest that the phosphatidylinositol (PtdIns)- and phosphatidylcholine-bound forms of Sec14 execute independent, yet complementary, functions dedicated to preserving a Golgi diacylglycerol (DAG) pool essential for Golgi-derived secretory vesicle biogenesis^{3,8,9}. We have solved a crystal structure for yeast Sec14 refined to 2.5 Å resolution (Table 1). The R -factor for the Sec14 model is 0.218 ($R_{\text{free}} = 0.273$) with good canonical properties. Sec14 crystal formation and maintenance required concentrations (1% w/v) of the non-ionic detergent *n*-octyl- β -D-glucopyranoside (β OG)¹⁰ that were in excess of its critical micellar concentration (0.74% w/v). The consequence was that the structure describes a β OG-bound form of Sec14.

Sec14 crystallized as a monomer with molecular dimensions of $60 \times 60 \times 50$ Å. The ordered component of the crystal structure comprises residues 4–299; thereby accounting for the entire functional core of Sec14. The structure consists of 12 α -helices (A1–A12), 6 β -strands (B1–B6) and 8 3_{10} (T1–T8) helices. Helices A1–A5 and T1 form the amino-terminal (N) domain, and α -helices A6–A12, β -strands B1–B6, and 3_{10} -helices T2–T8 form the carboxy-terminal (C) domain (Fig. 1a). The Sec14 N and C domains are held together by hydrophobic stacking interactions that involve residues W₈₂, Y₈₆ from A4; F₉₅ from A5; the hydrophobic arm of the

K₁₄₅ side chain from A8; and Y₂₀₄ from A9. A search for structurally homologous proteins in the Protein Data Bank (DALI 2.0 algorithm¹¹) did not reveal proteins with similar folds (Z -score > 3.5).

The N-domain α -helices A1–A4 are arranged essentially anti-parallel to each other, and helices A2, A3 and A4 form a tripod-like motif with a hydrophobic interior (Fig. 1a). This tripod encompasses Sec14 residues 32–86, and includes the bulk of the N-terminal 129 amino-acid residues that are sufficient for Sec14 targeting to yeast Golgi membranes^{8,12}. The 3_{10} -helix T1 and α -helix A5 cooperate to form a single helix that helps to link the Sec14 N and C domains tightly.

The Sec14 C domain is distinguished by a large hydrophobic pocket (volume = 3,000 Å³; Fig. 1a). The six β -strands (B1–B6) constitute the pocket floor, and α -helices A8 and A9 form one side of the cavity and A10/T4 and A11 form the other. β -Strands B2, B3, B4 and B5 orient parallel to each other and form a $\beta\alpha\beta\alpha\beta$ fold. β -Strands B1 and B6 are also arranged parallel to each other, but are in an antiparallel configuration relative to B2, B3, B4 and B5. Both helices A9 and A10/T4 are bent away from the pocket (and increase the dimensions of its mouth) and P₁₆₀ increases pocket volume by kinking α -helix A8.

Stabilization of the hydrophobic pocket is effected by Sec14 residues 243–299; a string-like motif that includes α -helix A12, β -strand B6, and 3_{10} helices T5, T6, T7 and T8. This motif extends around behind the β -sheet floor of the hydrophobic pocket and wraps it up (Fig. 1b). The four 3_{10} -helices within this region tighten the string. This string-motif provides a molecular basis for the *sec14-1ts* (temperature-sensitive) defect that renders Sec14 thermolabile for phospholipid transfer activity *in vitro* and for biological function *in vivo*^{13,14}. This allele represents a G266D mutation^{4,15} next to the 3_{10} -helix T5 (Fig. 1b). The structure reveals that the side chains of residues substituting for G266 point directly at T5 and that

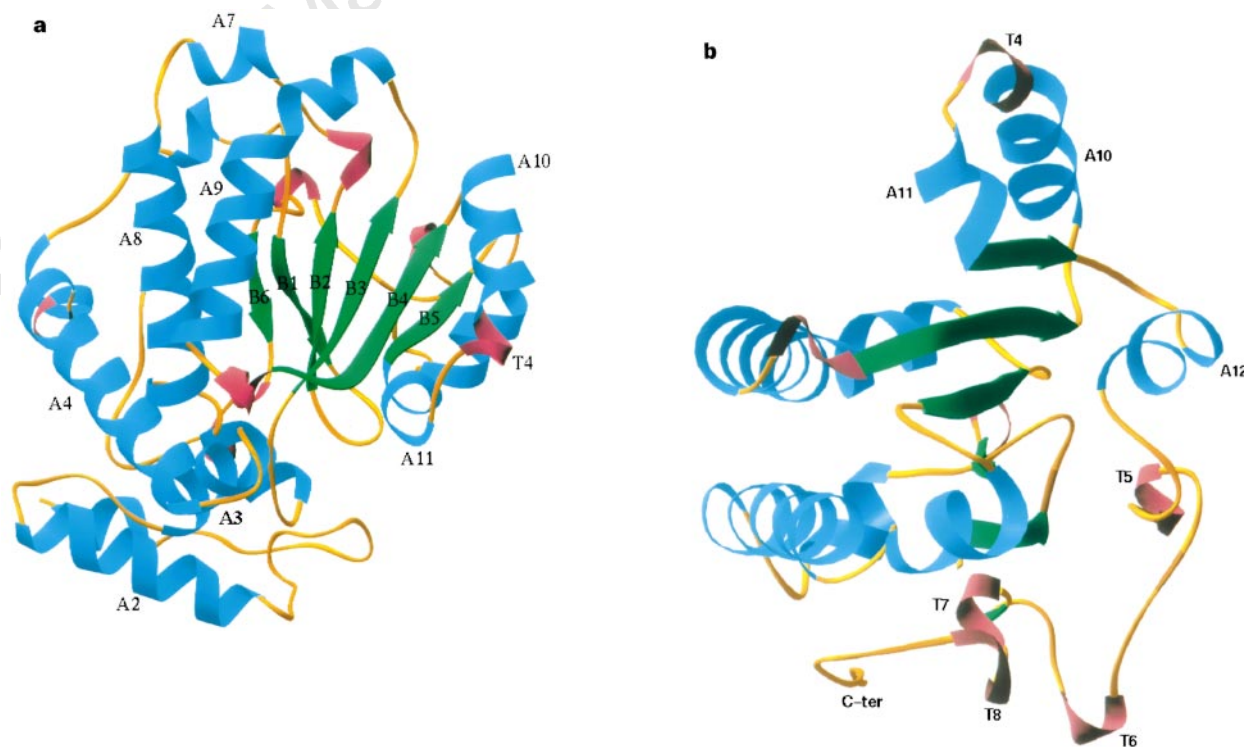


Figure 1 Sec14 structure. **a**, Ribbon drawing²⁷ of the Sec14 monomer structure. α -Helices (A1–A12, blue), β -strands (B1–B6, green), and 3_{10} helices (T1–T8, pink) are numbered from the N to the C terminus. Selected α -helices, 3_{10} helices and all β -strands are labelled and the hydrophobic pocket of Sec14 C-terminal domain is oriented so that it opens towards the reader. The two bound β OG molecules are

not shown. **b**, The Sec14 string motif. A ribbon diagram of residues 100–299 is shown. Relevant structures are labelled. The hydrophobic A10/T4 helix is swung approximately 10 Å units away from the protein surface. The string motif (comprising A12, B6 and the 3_{10} helices T5, T6, T7 and T8) extends around the rear of the Sec14 molecule and reinforces the hydrophobic pocket.

introduction of a large side chain at residue 266 will disturb the string motif by interfering sterically with helix T5. The consequence would be destabilization of the hydrophobic pocket. Mutagenesis data confirm both this structural prediction and the prediction that substitution of the adjacent G265 residue with large side-chain residues will not diminish Sec14 function.

α -Helices A3, A4 and A5 from the N domain may also contribute to stabilization of the hydrophobic pocket by surrounding and immobilizing C-domain α -helices A8 and A9 that form one side of the cavity. The N domain and the string motif exhibit greater flexibility than does the hydrophobic pocket, as the average B -factors of the main-chain atoms of the N domain ($59.65 \text{ \AA}^2$) and string motif ($41.03 \text{ \AA}^2$) exceed that of the hydrophobic pocket main-chain atoms ($34.75 \text{ \AA}^2$).

Two bound molecules of β OG were recognized in the Sec14 electron density map, and the detergent acyl chains extend away from the Sec14 interior whereas the headgroups are oriented inwards (Fig. 2a). The side chains of residues M177, L179 from B3, F212, I214 from B4, I240, I242 from B5, V190, M191, Y193, V194 from A9, and F221, F225, F228, L232 from A10/T4 line the pocket and render its upper region extremely hydrophobic. The detergent acyl chains make extensive van der Waals contacts with the hydrophobic pocket in this region, especially with helix A10/T4. The interfacial surface area between the detergent molecules and A10/T4 is $215 \text{ \AA}^2$. We propose that the acyl chains of Sec14-bound phospholipids contact these same hydrophobic surfaces. Because the dimensions of the Sec14 phospholipid ligands exceed those of

β OG, however, additional surface area within the hydrophobic pocket is probably involved in phospholipid binding. A10/T4 residues P218, F219, F221, A224, L227, F228, P230, F231 reside on the solvent-disposed face of the helix (Fig. 2a), and their hydrophobic side chains orient away from the protein surface. Thus, the solvent-exposed face of helix A10/T4 defines the most hydrophobic region of the Sec14 surface, the interior of the hydrophobic pocket included.

The displacement of bound phospholipid by β OG during Sec14 crystallization precluded direct identification of Sec14 residues that contact phospholipid headgroups. However, the β OG headgroup is chemically similar to inositol, and we identify six potential hydrogen bonds between Sec14 residues and the headgroup of β OG1 (Fig. 2b). Of these hydrogen bonds, the one between β OG1 O4 and E207 OE2 is the shortest, with an ideal hydrogen-bond distance between the two atoms ($2.505 \text{ \AA}$). Residues K239 and E207 form a salt-bridge which rotates E207 from the most favourable rotamer conformation to the observed orientation and facilitates formation of the hydrogen bond between β OG1 O4 and E207 OE2. Consequently, the structure predicts that the electrostatic interaction between the E207 and K239 side chains will be critical for PtdIns headgroup binding by Sec14. In support of this prediction: (1) both E207 and K239 are conserved in all Sec 14 homologues that exhibit PtdIns-transfer activity¹⁶, and (2) the K239A missense mutation abolishes Sec14 PtdIns-transfer activity *in vitro* without any effect on phosphatidylcholine-transfer activity (not shown).

Sec14-mediated phospholipid exchange is a remarkable reaction

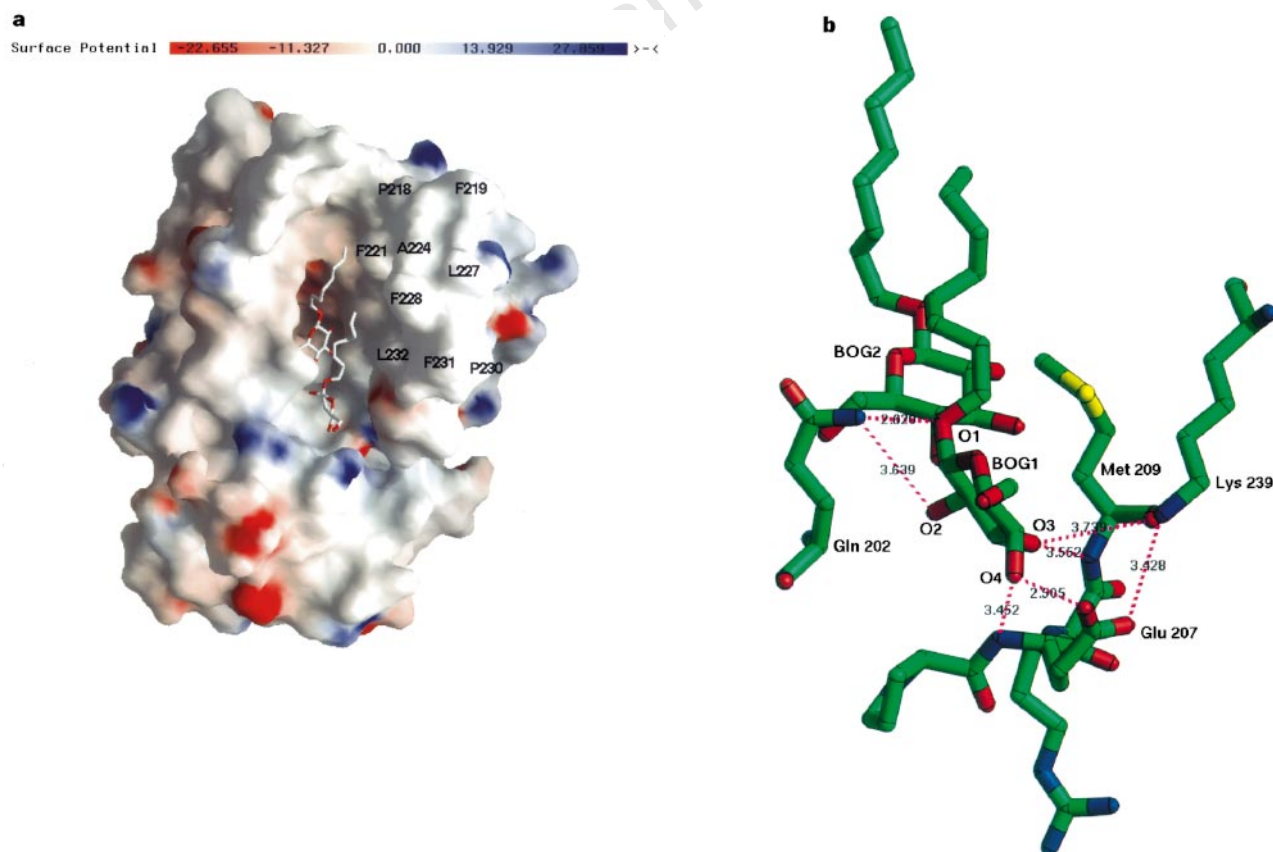


Figure 2 Bound detergent in the Sec14 structure. **a**, Orientation of bound β OG with respect to Sec14. A surface potential drawing of the phospholipid-binding region of Sec14 as determined by GRASP²⁸ is shown. Blue and red denote positively and negatively charged regions, respectively (scale of surface potential shown in colour bar). The hydrophobic pocket of Sec14 is oriented towards the reader, and the two bound β OG molecules are represented as ball and stick models. β OG1 is the frontmost and lower of the two detergent molecules. Hydrophobic residues that reside on the solvent-disposed face of helix A10/T4,

and whose corresponding side chains orient towards solvent, are also labelled. **b**, Interaction of Sec14 with detergent headgroup. The hydrogen-bond network through which Sec14 binds the β OG1 headgroup is illustrated. Hydrogen bonds are depicted by the dotted lines, and the corresponding atomic distances are given ($\text{\AA}$). Carbon atoms are depicted in green, nitrogen atoms in blue, sulphur in yellow, and oxygen atoms in red. Only those Sec14 residues involved in β OG1 headgroup binding are shown. The salt-bridge linking residues E207 and K239 is also labelled with a dotted line, and its atomic distance is provided ($\text{\AA}$).

that involves discharge of a bound phospholipid from Sec14 into the lipid bilayer, recognition of a resident phospholipid molecule by Sec14, and extraction of that resident phospholipid from the bilayer into the Sec14 interior. We propose that helix A10/T4 is critical for the ability of Sec14 to execute phospholipid exchange. Helix A10/T4 represents an interesting structural feature on three counts: (1) it protrudes away from the mouth of the hydrophobic pocket (Fig. 1b), (2) its solvent-disposed face defines the most hydrophobic Sec14 surface (Fig. 2a), and (3) it enters into extensive van der Waals contacts with the β OG acyl chains and presumably with the Sec14-bound phospholipid acyl chains as well. This configuration suggests

an A10/T4-driven 'bulldozer' mechanism for Sec14-mediated phospholipid exchange (Fig. 3).

These Sec14 data provide the first structural insights into the function of a phosphatidylinositol-transfer protein, and provide new information concerning the architecture of an entire family of evolutionarily conserved proteins. Of particular interest will be whether Sec14 and mammalian phosphatidylinositol-transfer proteins share structural homology, the absence of shared primary sequence homology notwithstanding^{12,17,18}.

Methods

Crystallization and data collection. The purification and crystallization of Sec14 has been described previously¹⁰. The native data set was collected at SSRL station 7-1 ($\lambda = 1.08 \text{ \AA}$) and processed by DENZO^{19,20}. The derivative data sets were collected using an in-house Siemens HSTAR multiwire area detector and processed by the X-GEN program.

Phase determination. The crystal structure was solved by multiple isomorphous replacement (MIR). Heavy-atom positions were located from isomorphous difference Patterson maps using the Xtalview 3.0 program²¹ and further refined by the MLPHARE program from the CCP4 package²². Solvent flattening ($V_m = 3.6 \text{ \AA}^3$, 65% solvent content in the unit cell, corresponding to one molecule per asymmetric unit²³) was performed using Solomon (CCP4).

Model building and refinement. Model building and adjustment were carried out by program O 5.10.3 using the $3 \text{ \AA}$ electron density map²⁴. Of 304 Sec14 amino acids, residues 4–299 were fitted into the electron density map followed by three cycles of torsion dynamics refinement by XPLOR 3.85 (ref. 25). Both R_{factor} and R_{free} were used as quality indicators during the refinement. Because of the large solvent content of the unit cell, use of a solvent mask during refinement substantially improved the R factors²⁶.

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Table 1 Structure determination and refinement

Processing statistics			
Data set	Native	K ₂ PtCl ₄	PbAc(CH ₃) ₃
Space group	P3221	P3221	P3221
a (Å)	88.971	88.951	88.823
c (Å)	111.339	110.798	111.507
Resolution (Å)	2.5	3.0	3.0
Unique reflections	16,736	10,230	10,984
R _{sym} (%)	5.1	6.0	5.8
Completeness (%)	90.8	93.0	99.8
R _{def} (%)		25.2	19.5
Phasing power		1.60	1.04
R _{cullis}		0.71	0.82
Combined figure of merit	0.496		
Refinement statistics			
Resolution range (Å)	30–2.5		
Number of used reflections	16,259 ($F > 2\sigma(F)$)		
R _{factor} (without sigma cutoff)	21.1% (21.1%)		
R _{free} (without sigma cutoff)	27.3% (27.3%)		
Number of model atoms	2,493 (53 water molecules)		
R.m.s. deviations from ideality			
Bond lengths (Å)	0.008	Bond angles (°)	1.422
Impropers (°)	1.218	Dihedrals (°)	24.085

$R_{\text{sym}} = \sum_i \sum_j |I_{hkl} - \langle I_{hkl} \rangle| / \sum_i \sum_j \langle I_{hkl} \rangle$, where hkl is the unique reflection index, I_{hkl} is the intensity of symmetry redundant reflections and $\langle I_{hkl} \rangle$ is the mean intensity. $R_{\text{def}} = \sum_i |F_{\text{PH}} - F_{\text{P}}| / \sum_i |F_{\text{PH}}|$, where F_{PH} and F_{P} are structure amplitudes of heavy-atom derivatives and native crystals, respectively.

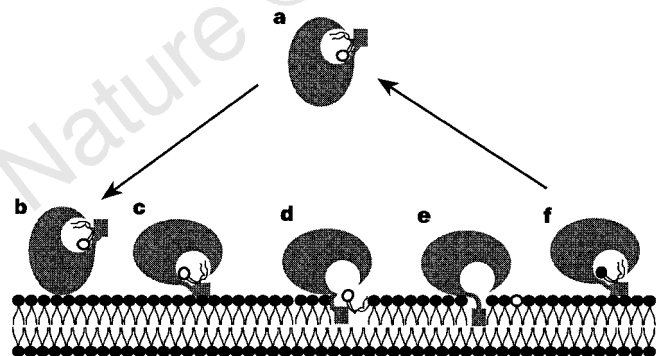


Figure 3 Bulldozer model for Sec14-mediated phospholipid exchange. A Sec14-phospholipid complex is stable in solution (a). Upon Sec14 (black oval) binding to Golgi membranes (b, c), helix A10/T4 (black square) swings away from the Sec14 surface and inserts into the lipid bilayer (d). Helix A10/T4 is positioned $10 \text{ \AA}$ away from the Sec14 surface (Fig. 1b) and may swing even further away. Assuming a bilayer thickness of $37 \text{ \AA}$ (ref. 29), A10/T4 can penetrate sufficiently deep to contact the acyl chains of outer leaflet phospholipids (e). During membrane insertion, A10/T4 drags a phospholipid molecule (in white) from the hydrophobic pocket and deposits it into the bilayer (d). During deinsertion, helix A10/T4 acts as a 'bulldozer' that abstracts another phospholipid molecule from the bilayer in the process of retracting towards the mouth of the unoccupied hydrophobic pocket (e–f). As A10/T4 retraction is probably slow relative to the very rapid rate of lateral diffusion of the newly deposited phospholipid monomer (translational diffusion coefficient = 10^{-9} – $10^{-8} \text{ cm}^2 \text{ s}^{-1}$; ref. 30), reabsorption of that same phospholipid molecule by Sec14 is unfavourable (e–f).

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Energy transduction in ATP synthase

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Mitochondria, bacteria and chloroplasts use the free energy stored in transmembrane ion gradients to manufacture ATP by the action of ATP synthase. This enzyme consists of two principal domains. The asymmetric membrane-spanning F_0 portion contains the proton channel, and the soluble F_1 portion contains three catalytic sites which cooperate in the synthetic reactions¹. The flow of protons through F_0 is thought to generate a torque which is transmitted to F_1 by an asymmetric shaft, the coiled-coil γ -subunit. This acts as a rotating 'cam' within F_1 , sequentially releasing ATPs from the three active sites^{1–5}. The free-energy difference across the inner membrane of mitochondria and bacteria is sufficient to produce three ATPs per twelve protons passing through the motor. It has been suggested that this protonmotive force biases the rotor's diffusion so that F_0 constitutes a rotary motor turning the γ shaft⁶. Here we show that biased diffusion, augmented by electrostatic forces, does indeed generate sufficient torque to account for ATP production. Moreover, the motor's reversibility—supplying torque from ATP hydrolysis in F_1 converts the motor into an efficient proton pump⁷—can also be explained by our model.

Figure 1a shows the molecular geometry of ATP synthase based on the available structural information⁵. Growing evidence indicates that the entire structure of ATPase can be divided into two counter-rotating assemblies (Fig. 1b). We shall call the assembly consisting of a, b, δ and the F_1 hexamer the 'stator', and place our coordinate system on it. The assembly consisting of c, γ and ϵ is the 'rotor'. Proton flow through the channels at the a–c interface generates torque which drives the rotor and stator in opposite directions. The motor must generate sufficient torque to produce three ATPs per revolution, an amount of work equivalent to $\sim 20 k_B T$ per ATP molecule (k_B is Boltzmann's constant and T the absolute temperature; at room temperature $1 k_B T \approx 0.6 \text{ kcal mol}^{-1} = 4.1 \text{ pN nm}$).

The central proton carrier in F_0 is Asp 61, which is found on the c subunit in 9–12 copies^{8,9}. Arg 210 is also an essential amino acid, and it resides on the a subunit in a single copy. (His 245 and Glu 219 are also involved, but may not be essential¹⁰.) These residues hold protons in an energy well whose depth is related to their pK_a values by $V/k_B T \approx -2.3 pK_a$. The sequence and topological arrangement of the amino acids in the a and c subunits have been determined, although their exact geometric configuration is uncertain^{3,5,11}. The

charge configuration we show here is the simplest geometry consistent with structural data; however, our mathematical description can accommodate other charge geometries^{12,13}. The geometrical relationship of the rotor Asp 61 sites and the stator Arg 210 site is shown in Fig. 2a. The key elements in this structure are: (1) the two half-channels that allow access of protons from the acidic and basic reservoirs to the rotor sites are offset, which confers an asymmetry on the proton flux. The notion of aqueous half-channels was first proposed for the bacterial flagellar motor^{14,15}. (2) The location of the positive stator charge between the half-channels is in a position to interact electrostatically with the rotor sites¹³.

The pK_a of Arg 210 is high enough that it is always protonated, and so its charge is fixed at +1. The rotor sites, however, have an intermediate pK_a , and so can be either protonated or not. When unprotonated, they cannot rotate out of the rotor–stator interface, for that would entail a large free energy penalty. Thus all rotor sites facing the bilayer must be protonated, and the membrane interface prevents any unprotonated rotor site from moving out the stator.

Arg 210 interacts with the rotor sites according to Coulomb's law in a dielectric and shielding environment typical of a protein interior (Table 1). If a rotor site passes close to the Arg 210 stator charge, the electrostatic interaction can reduce the rotor pK_a allowing the proton to dissociate. (This is because a protonated site is not exactly neutral, but constitutes a dipole whose strength is not sufficient to prevent it rotating into the bilayer but is strong enough to interact with a nearby charge.) The work of reducing a

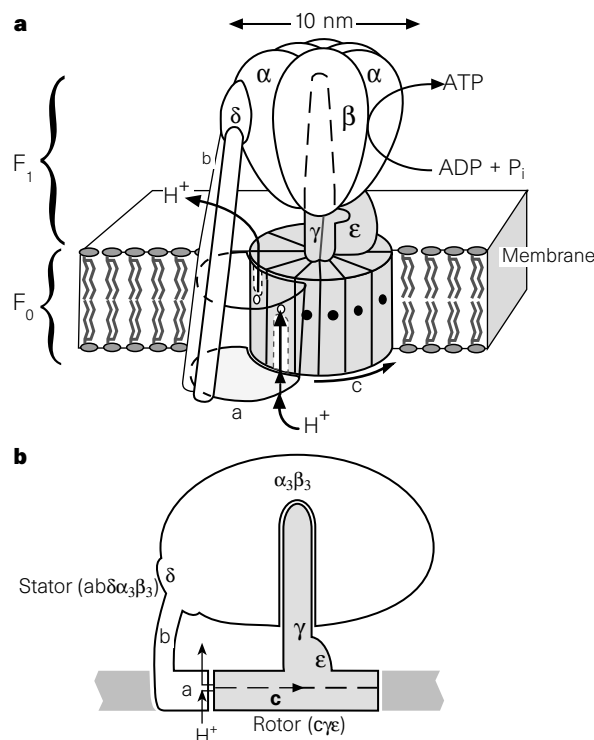


Figure 1 Structure of F_0F_1 ATP synthase^{5,6}. **a**, The c subunit consists of 9–12 twin α -helices arranged in a central membrane-spanning array⁸. The a subunit consists of 5–7 membrane-spanning α -helices. The proton channels lie at the interface between the a and c subunits (dashed lines indicate the putative inlet and outlet channels). The a subunit is connected to F_1 by the b and δ subunits. Proton flow through the channels develops torque between the a and c subunits. This torque is transmitted to F_1 via the γ shaft and the ϵ subunit, where it is used to release ATP sequentially from the three catalytic sites in F_1 . (Adapted from refs 4, 5.) **b**, Profile of the F_0F_1 structure showing the two counter-rotating structures. We put our coordinate system on the 'stator', which consists of the a, b, δ subunits and the F_1 hexamer. The 'rotor' is shaded, corresponding to the shaded region in **a**; it comprises the c, γ and ϵ subunits.

rotor site's pK_a values carries an energetic price: the proximity of a rotor and stator charge creates an electrostatic force between the rotor and stator that impedes its rotation.

The stator charges involved in proton transport are located on adjacent α -helices in the a subunit. Therefore, we shall assume that there are two rotor Asp 61 sites at the rotor/stator interface, as shown in Fig. 2a. We shall also assume that the rotor sites are accessible from the low- and high-pH sides through aqueous channels in the stator^{11,14,16}. Protons in the acidic reservoir have access to the right-hand rotor site and protons in the basic reservoir have access to the left-hand rotor site. We place the positively charged Arg 210 stator residue in a position midway between the two aqueous channels, and offset 0.52 nm out of the rotor plane.

Intuitively, the motor works as follows. First consider the situation when the positive stator charge is absent, which has been discussed elsewhere⁶. The two rotor sites within the stator can be protonated or unprotonated. When both sites are unprotonated, the rotor cannot diffuse in either direction, for that would entail moving an Asp 61 site into the bilayer. When the right-hand site is protonated, the rotor can diffuse to the right unimpeded, but is prevented from diffusing to the left by the other unprotonated site. Conversely, when the left site is neutralized by a proton, it can diffuse to the left, but not to the right. When both sites are neutralized, the rotor can diffuse freely in both directions. When the proton electrochemical potential difference across the membrane tends to drive protons upwards with reference to Fig. 2a, the rotor will diffuse preferentially to the right because the right-hand

site will be protonated more often than the left-hand site as it faces a higher proton concentration. Thus, on average, protons enter from the acidic reservoir, board the rotor and rotate to the right by one full revolution, then exit to the basic reservoir when they re-enter the stator from the left. However, this is not an efficient motor, for when both sites are occupied, the rotor will be forced backwards by the load torque, allowing protons to leak through to the basic reservoir without performing any work. Indeed, we will show that, without Arg 210, this biased diffusion cannot account for the observed properties of the motor.

Now consider the situation when the stator charge is present. When the right-hand site is protonated, half the time it will diffuse to the right and enter the membrane. This brings a corresponding charge into the stator from the left, where it can exit to the basic reservoir. However, when the rotor diffuses to the left and approaches the stator charge, its pK_a will drop and it will promptly relinquish its proton back to the acidic reservoir. Unprotonated, the bare site is quickly pulled into apposition with the stator charge and held in place against the load torque. However, a thermal fluctuation can carry the site away from the stator charge far enough to reprotonate from the acidic channel, whereupon the site gets another try at diffusing to the right. The same cycle of events takes place at the basic channel, but owing to the proton gradient, the rotor's diffusion is consistently biased to the right. The presence of the positive stator charge prevents back-diffusion of the rotor and couples the proton flux tightly to the rotor motion. This more than compensates for the retarding effect of the electrostatic interaction,

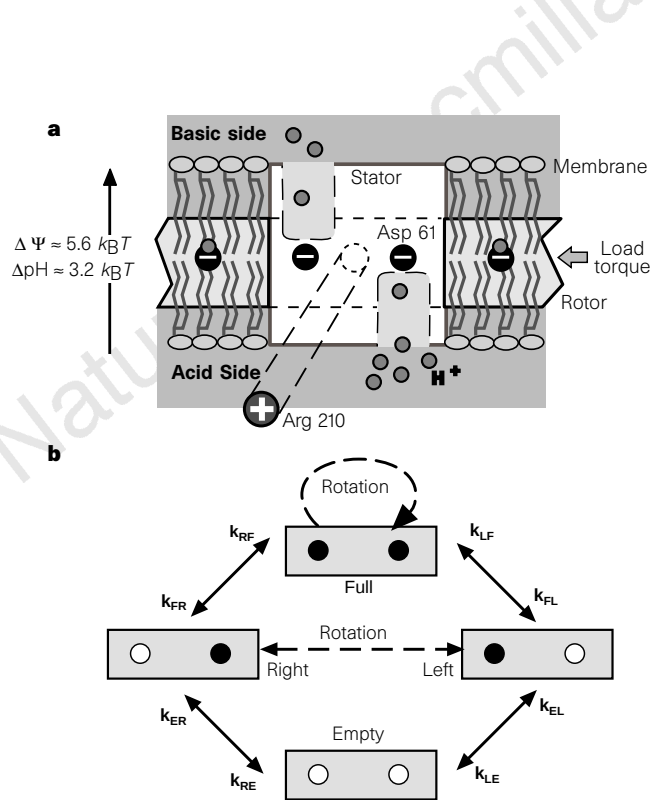


Figure 2 Charge geometry and movement of protons in ATP synthase. **a**, Face-on view showing the relative locations of the stator and rotor charges and the proton channels. The two proton reservoirs are connected by offset 'half-channels' which confer an asymmetry on the assembly^{6,14}. Arg 210 is located on the a subunit in a plane 0.52 nm offset from the plane of the rotor. **b**, Markov chain describing the four possible motor states and their transition rates, $k_{i \rightarrow j}$. E, empty; F, full; L, left site occupied; R, right site occupied. White circles, unprotonated Asp 61 sites; black circles, protonated sites.

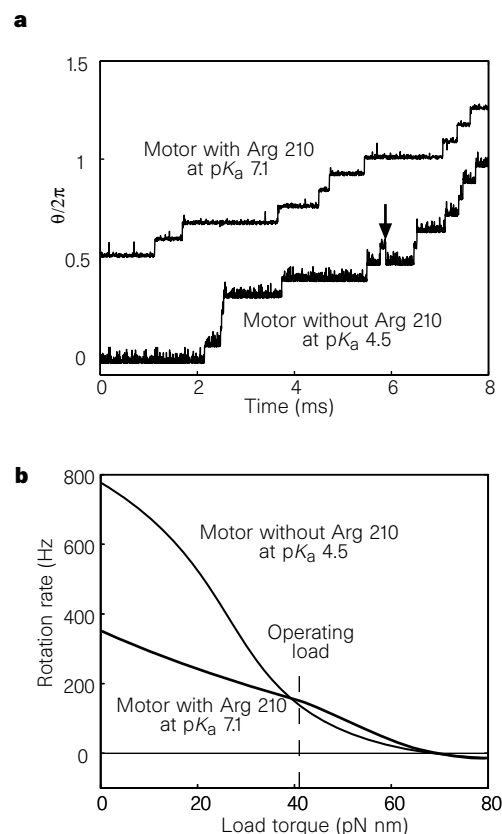


Figure 3 Properties of the ATP synthase rotor. **a**, Stochastic simulation of the rotor motion when a protonmotive force of 220 mV is imposed across the membrane and a load torque of 41 pN nm resists the motion. In the absence of Arg 210, the rotor advances in steps, with occasional reversals (arrow), when protons can leak through the stator without advancing the rotor. When Arg 210 is present, the motor suffers almost no reversals, and the amplitude of its stochastic fluctuation is smaller. **b**, Angular velocity plotted against load torque for the motors with and without Arg 210.

and enables the engine to work more efficiently as both a motor and pump. To justify this qualitative description of the motor's operation, we must write and solve the equations governing the rotor motion. Various aspects of this mechanism have already been discussed qualitatively^{4,13,17–19}.

Proton motions are much faster than rotation of the rotor. This enables us to model the movements of the protons by transitions between a set of discrete states (that is, a Markov chain²⁰). As each site can be protonated or not, there are four possible protonation states of the assembly, denoted by $s = R$ (right), L (left), E (empty), F (full), as in Fig. 2b. Each time a proton hops into or out of the stator, or the rotor moves, the state switches, and in each state the rotor experiences different torques.

We can neglect inertia and describe the rotation of the rotor by a torque balance that includes thermal fluctuations²¹:

$$\underbrace{\zeta 2\pi\Omega(\theta)}_{\text{viscous drag torque}} = \underbrace{F_E(\theta, s)}_{\text{electrostatic torques from the stator}} + \underbrace{F_H(\theta, s)}_{\text{hydrophobic torque from the membrane}} - \underbrace{\tau}_{\text{load torque from } F_1} + \underbrace{f(t)}_{\text{brownian torque}}, s = R, L, E, F \quad (1)$$

Here $2\pi\Omega = d\theta(t)/dt$ is the angular velocity of the rotor, ζ is the drag coefficient of the rotor, τ is the load torque from F_1 , and $f(t)$ is the brownian torque from thermal fluctuations. $F_E(\theta, s)$ is the electrostatic torque acting on the rotor from the interaction between rotor charges and Arg 210. The hydrophobic force, F_H , arises from the potential barrier preventing the motion of an unprotonated site into the membrane; this energy barrier is $\sim 45 k_B T$. To compute the torque generated by the motor, equation (1) must be solved simultaneously with the Markov process governing the hopping of protons on and off of the rotor sites.

The model equations were solved numerically using the parameter values listed in Table 1; the details of all computations are given in Supplementary Information. Figure 3a shows a stochastic simulation of the rotor motion when the proton flow through the stator is driven by a protonmotive force of $\Delta p \sim 220$ mV and the rotor works against a constant load of 41 pN nm, which is equivalent to the torque necessary to produce three ATPs from F_1 per rotation. We see that the motor progresses stepwise, each step corresponding to the passage of one proton through the motor.

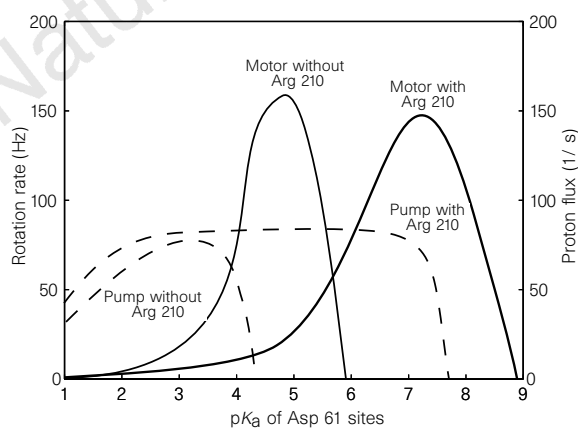


Figure 4 Solid lines show the rotation rate of the motor as a function of the pK_a of the rotor Asp 61 sites with and without the presence of the stator Arg 210 charge when the load torque is fixed at 41 pN nm. The optimum pK_a for the motor operating without Arg 210 is 4.5–5; in the presence of Arg 210, the optimum is 7–7.5. Dashed lines show the reverse proton flux when the motor operates in reverse as a proton pump. $200 k_B T s^{-1}$ is supplied by F_1 to rotate the γ -subunit. In the absence of Arg 210, the optimal pK_a is 3–3.5, whereas in the presence of Arg 210, the optimum is 6–7. These plots show that Arg 210 is necessary for F_0 to function as both a motor and a pump.

On average, 12 protons pass through the motor per rotation, corresponding to 4 protons per ATP. The rotation is tightly coupled to the proton flux because the presence of Arg 210 prevents leakage of protons by back-rotation. Figure 3b shows a load–velocity curve for the F_0 motor. As rotation is tightly coupled with proton flow, the motor efficiency increases roughly in proportion to the load. When rotating against the ‘normal’ load torque from F_1 , the motor is operating at $\sim 60\%$ efficiency. It is likely that the load from F_1 is not constant, so several other load patterns were investigated: our general conclusions were unaltered.

The crucial role of electrostatic forces can be demonstrated quantitatively by computing the motor performance with and without the stator Arg 210 residue. In Fig. 4 we have plotted the rotation rate of the motor as a function of the pK_a of the rotor sites. In the presence of Arg 210, the optimal motor performance is close to the measured pK_a value of 7.1 (ref. 22). In the absence of the stator charge, the motor cannot develop any torque above $pK_a \sim 6$, and is completely decoupled from the proton flux. To work without the stator charge, the rotor pK_a must be lowered to 4.5–5, close to its solution value. In Fig. 3a we have plotted a stochastic simulation of the motor in the absence of Arg 210 at a rotor pK_a of 4.5. It operates quite well, but suffers occasional reversals because it cannot resist the load torque in the doubly occupied F state. Thus without the electrostatic coupling induced by Arg 210, the motor is not as tightly coupled to the proton flux. Moreover, to produce sufficient torque at this low pK_a the entry rate of protons must be at least $10^4 s^{-1}$, because most protons promptly dissociate back into the acidic reservoir (see Supplementary Information).

There is an additional constraint on the motor's performance. Under anaerobic conditions, the ATP synthase of *Escherichia coli* reverses, using ATP hydrolysis to pump protons against a pH gradient. To investigate the performance of the motor as a pump, we assumed an ATP hydrolysis rate by F_1 of 50 ATP molecules per second²³, an efficiency of 20% in converting this to rotary torque (corresponding to a power input of $2\pi\Omega\tau$), and a gradient of 1 pH unit. The basic reservoir was maintained at a pH of 7.6 as this is the value at which *E. coli* maintains its cytoplasm. Figure 4 shows that, in the absence of Arg 210, the optimal pK_a of the rotor sites is 3–3.5, quite far from the optimal operating region for the motor. Above $pK_a \approx 4.3$, the motor can no longer act as a pump. Thus without Arg 210, the motor is a poor proton pump. However, in the presence of Arg 210, it performs quite well as a proton pump near the optimal pK_a for the motor. Therefore, omitting the electrostatic forces in equation (1) prevents the motor from operating efficiently as both a motor and pump. Moreover, the many protonation–deprotonation

Table 1 Parameter values used in calculations

Parameter	Value
Proton diffusion coefficient (D_p)	$9.3 \times 10^8 \text{ nm}^2 \text{ s}^{-1}$
Rotary diffusion coefficient of the rotor (D_r)	$2 \times 10^4 \text{ s}^{-1}$
Dielectric constant of channel (ϵ_c)	10
Dielectric constant of the membrane (ϵ_m)	3
Bilayer viscosity (η)	1 poise
Height of rotor (h)	6 nm
Shielding length of channel charges ($1/\lambda$)	1.1 nm
pH^A (pH of acidic reservoir)	motor, 7; pump, 6.6
pH^B (pH of basic reservoir)	motor, 8.4; pump, 7.6
‘Radius’ of the proton channel (r)	0.5 nm
Radius of rotor (R)	5 nm
Distance (Δx) between Asp 61 residues, $2\pi R/12$	2.6 nm
pH difference across the rotor (ΔpH)	80 mV = $3.2 k_B T$
Membrane potential ($\Delta\psi$)	140 mV = $5.6 k_B T$

events that occur before a successful rotor movement require a high entry rate for protons in order to generate sufficient torque.

The average electrostatic torque felt by the rotor opposes the rotor's motion, so that the field actually works against the motor. However, this energy penalty is more than compensated for by two beneficial effects: (1) the high pK_a of the rotor sites holds protons tightly and prevents futile protonation-deprotonation cycles: this increases the effective proton supply to the stator; (2) by forcing the rotor site to relinquish its proton when passing the stator charge, the stator charge tightly couples the proton flux to the rotor motion. This increases the effectiveness of rectifying the rotor's diffusion, and more than compensates for the additional work done by the protonmotive force against the electric field.

We have presented a mechanism for transducing free energy stored in an ion gradient into a rotary torque. Our analysis delineates the quantitative conditions under which the structure shown in Fig. 1 can actually generate the power required for ATP synthesis. This mechanism has several attractive properties. First, the motor can be reversed to form a proton pump, as in *E. coli* and the V-ATPase proton pumps²⁴. Second, the motor works as well when other ions are substituted for protons: for example, the F_0F_1 ATPase of *Progenium modestum*, which is very similar in structure to ATP synthase but operates on sodium ions rather than protons²⁵. Finally, the model provides an explanation for the effects of mutations in the crucial rotor and stator amino acids¹⁰. In addition to elucidating the mechanisms of torque generation, the model provides a framework for integrating the kinetic, thermodynamic and mechanical aspects of protonmotive energy transduction. □

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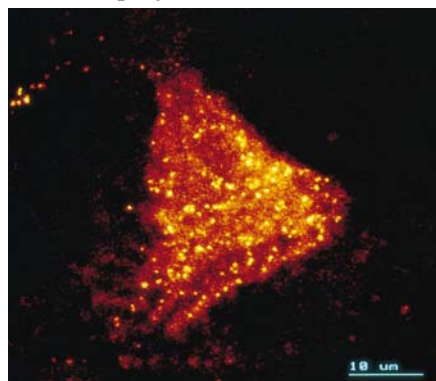
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From Semat

Two newly identified endogenous peptides: endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂)

These Tocris manufactured peptides differ by only one amino acid and have been found to have a high affinity for μ -opioid receptors, showing very high selectivity over other opioid receptor subtypes. Zadina *et al.* (*Nature* 386, 499; 1997) described these peptides as having the highest specificity and affinity for the μ -opioid receptor of any endogenous substances so far described. The manufacturer offers these endomorphins with a stated purity of more than 99 per cent in 1-mg or 5-mg amounts.

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PeptiKit

From Altus

A product to aid in the rapid determination of the optimal catalyst in a variety of peptide or peptide-related coupling reactions

The kit allows chemists to couple a broad range of amino acids and peptide fragments and their derivatives. It is designed to provide an efficient means of screening the 15 commercially available catalysts and determining the optimal synthetic route for a compound. With this system, users should be able to determine in 24 hours whether a peptide or peptide-mimic coupling reaction can be performed. The advantage of this product is that it will allow customers to quickly simplify catalyst identification, chemical scale-up and significantly reduce chemical wastes generated by traditional large-scale peptide and peptidomimetic production techniques. PeptiKit consists of two components: PeptiScreen for identifying the right catalyst for a peptide coupling reaction and PeptiTool for optimizing the reaction yield and time.

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Software

Amber 5.0

From Oxford Molecular Group

A program for simulating peptide, protein and DNA structures and their interactions

This program represents a set of simulation tools used in drug discovery. Developed at the University of California, San Francisco, version 5.0 includes functions that have been parallelized to speed up the calculation process. There is also a function for pictorial representation of free energy



Inside the Amber 5.0 software, GCG's peptide, protein and DNA interaction simulation system.

changes, which allows users to look for improved binding by rapidly screening the free energy effects of modified chemical groups. The realism of simulations of solvated DNA and protein/DNA have been improved in the new parallelized implementation of the particle-mesh, Ewald routine. For the first time, stable simulations can be carried out without artificial constraints, says the manufacturer. Enzyme reaction mechanisms, which involve bond-breaking processes and unusual cofactors or metals, can now be addressed by incorporating a quantum mechanical description of an active site. Other enhancements include new parallelized methods for determining protein and nucleic acid structures from NMR data. These methods apply to paramagnetic metallo-enzymes, as well as diamagnetic systems. The use of locally enhanced sampling in dynamics simulations can determine the global energy minima of solvated systems in a viable fashion.

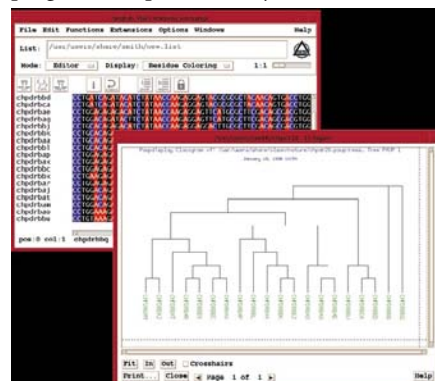
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Wisconsin Package

From Genetics Computer Group (GCG)

Version 9.1 — the next step for the group's sequence analysis package

This newest release is said to deliver new programs for protein analysis and the exclu-



GCG's SeqWeb interface displays a phylogenetic tree from a multiple sequence alignment.

sive distribution (on UNIX and OpenVMS systems) of the PAUP evolutionary analysis software package, authored by David Swofford of the Smithsonian Institution. There are two new protein analysis programs to help researchers understand the secondary structure and function of protein molecules: CoilScan predicts amino acid residues involved in coiled-coil structures; and HTHScan predicts helix-turn-helix motifs. A third new protein analysis program, SPScan, identifies secretory signal peptides in proteins destined for translocation across cellular membranes. The Wisconsin Package and SeqLab, an interactive sequence editor and interface to the Package, provides over 120 DNA and protein analysis programs. One of the package's strengths is its access to a number of genetic databases and its powerful database searching capabilities. The software runs on Digital AXP, IRM RS/6000, Silicon Graphics and Sun computers.

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Equipment and reagents

Filtron brand stirred cell systems

From Pall Gelman Sciences

To facilitate convenient processing of proteins or other biomolecules

With high-performance membranes that are ultrasonically sealed into 10- or 150-ml cells, these systems eliminate the need for individual membrane discs. Moreover, there is no O-ring, which eliminates this as a source of solution bypass or cross-contamination. These systems use highly porous membranes to provide high flow rates and recoveries. The devices are offered with a low protein-binding Omega membrane, available in 15 molecular weight cut offs or pore sizes. A Nova membrane is also offered, which is available in eight molecular weight cut offs. The cells consist of only three components for easy assembly and, as self-contained cells, they can be cleaned and reused, or disposed of when working with biologically hazardous or radioactive materials. Stirred-cell systems are well suited for concentration, fractionation, purification, buffer exchange or desalting of protein solutions, cell broths and other biomolecules.

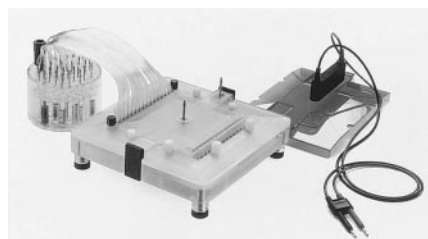
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Whole-gel eluter

From Bio-Rad

Provides automatic fractionation of protein bands without having to slice up the gel

This system automates the harvesting of protein bands from cell extracts separated by polyacrylamide gel electrophoresis. It collects all the proteins from an entire uncut gel, placing the content of each band into a separate well for subsequent sequencing, assay or antibody production. The instrument is well



Picky about bands? Try Bio-Rad's whole-gel eluter.

suited for screening milligram amounts of protein mixtures in cellular assays, or for characterizing T-cell and antibody responses. Antigenic fractions are obtained in non-toxic form, making the assays fast and easy to perform. Gels are transferred directly to the whole-gel eluter and an electrical field is applied. Protein fractions are then drawn through the gel thickness into closely spaced, machined channels under the slab. These can then be collected either manually or with the aid of an optional vacuum-assisted harvester. The whole elution process is stated to take from 14–21 min on a 3-mm thick gel, which should prove to be much faster than physically slicing the gel up into sections to extract each band. Two versions of the instrument are available. The full whole-gel eluter accepts gel sizes of the standard 14 cm × 16 cm (or larger gels cut to size), collecting 30 3-ml protein fractions (bands 6 mm apart can be isolated in pure form). The mini eluter is intended for studying smaller amounts of protein, taking slabs of 6.5 cm × 5.5 cm or larger, yielding up to 14 eluents of 0.5 ml each with a band resolution of 5 mm.

Reader Enquiry No. 107

Tween-detergents

From Sigma

Two new low-peroxide, low-carbonyl detergents that protect sensitive proteins against the undesirable actions of peroxides and carbonyl compounds

Specially purified and packaged under an inert atmosphere, Tween 20R (polyoxyethylenesorbitan monolaurate) and Tween 80R (polyoxyethylenesorbitan monooleate) provide consistent results without altering protein function. The products are suited for isolating, purifying and preserving non-specific binding proteins, manufacturing recombinant proteins, solubilizing specimens, maintaining reagents in solutions and performing lysis of cells. These detergents are formulated to protect sensitive proteins from oxidation and denaturation. The total peroxide for each is certified at $\leq 0.5 \mu\text{mol g}^{-1}$; whereas, carbonyls are certified at $\leq 1.0 \mu\text{mol g}^{-1}$ for each. To promote long shelf life without peroxide formation, both detergents have also been stabilized with an anti-oxidant. They are available in undiluted 10-ml and 100-ml bottles, permitting easy

dilution to desired concentrations without waste. Sigma provides over a dozen commercial Tween products.

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Amino acid derivatives

From Genzyme Pharmaceuticals

A selection of amino acid derivatives and related products for solid- and solution-phase synthesis of peptides and peptidomimetics

The product line comprises amino acids protected with Fmoc (9-fluorenylmethoxycarbonyl), Z-(benzyloxycarbonyl) and Boc (t-butyloxycarbonyl), some of which are N-methylated. Peptide linkers are also available for solid-phase synthesis. Genzyme also offers custom manufacture of amino acid derivatives and peptide fragments required in pharmaceutical development projects. The product range is available as stock or prepackaged, as well as in custom manufactured multi-kilogram to ton quantities. The product line is packaged in either polyethylene containers with tamper-evident aluminum seals or in fibreboard drums with double tamper-evident seals.

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